Olfactory neuroethology of the Oriental fruit moth, Grapholita molesta (Busck)

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Olfactory neuroethology of the Oriental fruit moth, *Grapholita molesta* (Busck)

-from Behavior to ORNs

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Ph.D. dissertation

to obtain a doctoral degree in Ciencia i Tecnologia Agraria i Alimentaria

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Affectionately dedicated to

The scientific community
César Gemeno
and my Family

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Contents

Publications

Directly involved in the Ph.D. dissertation

1) Ammagarahalli, B., Gemeno, C., 2014. Response profile of pheromone receptor neurons in male *Grapholita molesta* (Lepidoptera: Tortricidae). Journal of Insect Physiology, 71, 128-136.

2) Ammagarahalli, B., Gemeno, C., 2015. Interference of plant volatiles on pheromone receptor neurons of male *Grapholita molesta* (Lepidoptera: Tortricidae). Journal of Insect Physiology, 81, 118-128.

3) Ammagarahalli, B*., Barros-Parada, W*., Basoalto, E., Levi, A., Fuentes-Contreras, E., and Gemeno, C., Low reproducibility of attraction to plant lures in the moth *Grapholita molesta* (Lepidoptera: Tortricidae). Entomologia Experimentalis et Applicata (*Submitted*)

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4) Álvarez, G*., Ammagarahalli, B*., Hall, D.R., Pajares, J.A., Gemeno, C., 2015. Smoke, pheromone, and kairomone olfactory receptor neurons in males and females of the pine sawyer *Monochamus galloprovincialis* (Olivier) (Coleoptera: Cerambycidae). Journal of Insect Physiology, 82, 46-55.

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Abstract

The olfactory system has become an important model system for the study of sensory processing. Unlike vision in human beings, insects' activity is strongly associated with odours released by conspecifics, heterospecifics, host plants, etc. Under natural conditions, both plant and pheromone odors occur simultaneously, and insects exploit these odors to locate resources. Plant odors are known to interfere withinsect pheromone communication either by masking or enhancing its detection. The knowledge of odours (pheromones and plant odors) modifying the behaviors of insects allow us to use them directly or indirectly in integrated pest management. The oriental fruit moth, *Grapholita molesta* (Busck) (Lepidoptera: Tortricidae) is an important pest in stone fruit crops. Female *G. molesta* emit a three-component pheromone blend composed of (*Z*)-8 dodecenyl acetate (*Z*8-12:Ac), (*E*)-8 dodecenyl acetate (*E*8-12:Ac), and (*Z*)-8 dodecenyl alcohol (*Z*8-12:OH), at a ratio of 100:6:10, respectively. Male moths' activity is guided by these molecules in a plant odor background. Synthetic pheromone blend is used in pest management. Both sexes exploit plant odors to find their host plants, and males to find their conspecifics. The main objective of this dissertation was to gain insight on the olfactory neuroethology of *G. molesta* to pheromone and plant odors through behavioral and physiological studies.

Three different plant blends (Australian, Chinese, Swiss) were tested in the wind tunnel and synergised male responses to a suboptimal under-dose pheromone concentration. In addition, the mixture of pheromone and plant odors decreased the time it took males to engage in the flight responses, compared with pheromone alone. In contrast, these blends decreased pheromone captures in the field, and did so in a dose-dependent manner. On the other hand, plant blends alone showed no attraction of *G. molesta* in either laboratory or field conditions. Male attraction was lower to a suboptimal overdosed pheromone dose than to the optimal dose, and its combination with plant blend did not improve male flight responses. The ratio of two acetate components in the pheromone blend is critical for male attraction. Interestingly, the plant blend improved male flight responses to a pheromone blend containing unnatural ratio of the two acetates.

The role of *Z*8-12:OH in the pheromone blend is not completely clear. I retested the role of *Z*8-12:OH and related alcohols, and their interplay with plant volatiles. 12:OH (a proposed pheromone ingredient of *G. molesta*) and *E*8,*E*10-12:OH (codlemone, the sex pheromone of *Cydia pomonella*) supplanted the role of Z8-12:OH when this compound was removed from the blend. This shows that several chemically related alcohols can play the same role as the sex pheromone alcohol. But even more interesting was the fact that the plant blend could also substitute the absence of the alcohol.

Pheromone synergism most probably starts at the central nervous system level, but some studies in moths show that it can already start with interactions between pheromone and plant odors occurring at the pheromone olfactory receptor neuron (ph-ORN) level. In order to explore this possibility I first characterized the morphology of sensilla with scanning electron microscopy and the electrophysiological response of olfactory receptor neurons of *G. molesta* males with extracellular electrophysiology. 72%

were sensilla trichodea and housed ph-ORNs. The main pheromone components, *Z*8-12:Ac and 12:Ac were detected by highly specialized ORNs, and their proportion on the antennae (100:11.6, respectively) was similar to their ratio in the blend (100:6, respectively). No ORN was tuned to the minor component (*Z*8-12:OH). The response of Z-ORNs was very specific, whereas E-cells also responded to the Z isomer, albeit with lower sensitivity. About 30% of the ORNs in sensilla trichodea did not respond to any pheromone components tested, but some of them were tuned to plant odorants. Plant odors were detected by a different class of olfactory receptor neurons with various degrees of specialization and were housed in sensilla trichodea and auricillica. Stimulation of Z-ORNs with binary mixtures of *Z*8-12:Ac and biologically relevant doses of plant odorants in increasing doses slightly decreased their response to sex pheromone. The response of *E*-ORNs to a combination of *E*8-12:Ac and plant volatiles was not different from *E*8-12:Ac alone. Stimulation with plant blend alone did not change the firing rate of Z- and E-ORN types. I conclude that the observed behavioral pheromone-plant synergism could occur in the antennal lobe neurons like in other moths, but it is unlikely due to the small effect found. The findings presented in this thesis widen the knowledge of behavior and neuronal mechanisms to pheromone and plant odors in male *G. molesta*.

Keywords: *Grapholita molesta*, olfaction, single sensillum recording, sex pheromone, plant volatiles, flight tunnel, electrophysiology.

Resum

El sistema olfactiu s'ha convertit en un important sistema model per al processament sensorial. A diferència de la visió en els éssers humans, l'activitat dels insectes s'associa principalment amb les olors provocats ja sigui per la seva congèneres, heterospecifics, o pels volàtils emesos per les plantes hospederas. En condicions naturals, tots dos olors de plantes i de feromones es produeixen simultàniament, i els insectes exploten aquestes olors. Olors de plantes són coneguts per interferir en la comunicació de feromones ja sigui emmascarant o millorar la detecció de feromones en insectes. El coneixement de les olors (feromones i olors de plantes) que modifiquen el comportament dels insectes ens permet utilitzar directament o indirectament en la gestió integrada de plagues. L'arna oriental de la fruita, *Grapholita molesta* (Busck) (Lepidoptera: Tortricidae) és una plaga important en els cultius de fruites d'os. Dona G. molesta emeten una barreja de tres components de feromona composta d'acetat de (Z) -8 dodecenilo (*Z*8-12: Ac), (E) -8 acetat de dodecenilo (*E*8-12: Ac), i (Z) -8 dodecenilo alcohol (*Z*8-12: OH), en una proporció de 100: 6: 10, respectivament. Activitat de les arnes masculines "es guia per aquestes molècules en un fons olor planta. Barreja de feromona sintètica s'utilitza en el maneig de plagues. Tots dos sexes exploten les olors de plantes per trobar la seva planta hoste i homes per trobar als seus congèneres. L'objectiu principal d'aquesta tesi era per guanyar la penetració en el neuroetologia olfactiva de *G. molesta* feromones i vegetals olors a través d'estudis de comportament i fisiològiques.

Tres barreges de plantes diferents (australians, xinesos, suïssos) van ser provats en el túnel de vent i sinergizados respostes masculines a una concentració de feromona subòptima sota-dosi. En addició, la mescla de feromona i vegetals olors disminuir el temps que va prendre mascles per participar en les respostes de vol, en comparació amb la feromona sola. Per contra, aquestes barreges van disminuir captures de feromones al camp, i ho van fer d'una manera dependent de la dosi. D'altra banda, la planta mescles sol, no va mostrar atracció de *G. molesta*, ja sigui en condicions de laboratori o al camp. Home atracció es va reduir a unes dosis subòptimes de feromones sobredosi, i la seva combinació amb la barreja de plantes no va millorar les respostes de vol de sexe masculí. La proporció d'acetat de dos components en la barreja de feromona és crític per a l'atracció de mascles. Curiosament, la barreja de la planta va millorar respostes vol masculins a una barreja de feromones que conté la relació natural dels dos acetats.

El paper de *Z*8-12: OH en la barreja de feromones no és del tot clara. Jo a realitzar la prova el paper de Z8-12: alcohols OH i relacionats, i la seva interacció amb els volàtils de plantes. 12: OH (un ingredient feromona proposta de *G. molesta*) i *E*8, *E*10-12: OH (codlemona, el sexe phermone de *Cydia pomonella*) suplantat el paper de *Z*8-12: OH quan aquest va ser retirat de la mescla. Això demostra que diversos alcohols químicament relacionades poden jugar el mateix paper. Però encara més interessant va ser el fet que la barreja planta també podria substituir l'absència de l'alcohol.

Sinergisme Feromones molt probablement s'inicia a nivell del sistema nerviós central, però alguns estudis en arnes mostren que ja pot començar en les interaccions que ocorren entre feromones i vegetals olors en la neurona olfactiva del receptor de feromones nivell (ph-ORN). Per explorar aquesta possibilitat per primera vegada va caracteritzar la

morfologia de microscòpia electrònica d'escombrat wih sensilla i la resposta electrofisiològica de les neurones olfactives del receptor de mascles *G. molesta* amb l'electrofisiologia extracel·lular. El 72% eren trichodea sensilla i allotjats ph-ORNs. Els principals components de la feromona, *Z*8-12: Ac i *E*8-12: Ac van ser detectats per ORNs altament especialitzats, i la seva proporció en les antenes (100: 11,6, respectivament) va ser similar a la seva proporció en la mescla (100: 6, respectivament). No ORN estava sintonitzada en el component minoritari (*Z*8-12: OH). La resposta de Z-ORNs va ser molt específic, mentre que E-cèl·lules també van respondre a l'isòmer Z, encara que amb menor sensibilitat. Al voltant del 30% dels ORNs en trichodea sensilla no va respondre a cap dels components de feromones analitzades, però alguns estaven en sintonia amb olors vegetals. Les olors de la planta van ser detectats per una classe diferent de les neurones olfactives del receptor amb diferents graus d'especialització i van ser allotjats en trichodea sensilla i auricillica. L'estimulació Z ORNs amb mescles binàries de *Z*8-12:Ac i dosis biològicament rellevants d'olors de plantes en dosis creixents disminuir lleugerament la seva resposta a la feromona sexual. La resposta d'E-ORNs a una combinació de E8-12: Ac i vegetals volàtils no era diferent de *E*8-12:Ac sol. Estimulació de la barreja de plantes soles no va canviar la taxa de trets de Z i E ORN tipus. La meva conclusió és que el comportament sinergisme feromona vegetal observada podria ocórrer en les neurones del lòbul antenal com en altres arnes. En conclusió, els resultats presentats en aquesta tesi ampliar el coneixement del comportament i els mecanismes neuronals de feromones i vegetals olors en masculí *G. molesta*.

Paraules clau: *Grapholita molesta,* olfacte, gravació sensillum sola, feromones sexuals, volàtils de plantes, túnels de vol, electrofisiologia.

Resumen

El sistema olfativo se ha convertido en un importante sistema modelo para el estudio del procesamiento sensorial. A diferencia de la visión en los seres humanos, la actividad de los insectos se asocia principalmente con los olores provocados por miembros de la misma u otras especies, plantas huésped, etc. En condiciones naturales ambos olores se producen simultáneamente y los insectos explotan estos olores. Los olores de planta interferfieren en la comunicación con feromonas ya sea enmascarando o mejorando la detección de feromonas en insectos. El conocimiento de los olores (feromonas y olores de plantas) que modifican el comportamiento de los insectos se puede usar directa o indirectamente en la gestión integrada de plagas. La polilla oriental de la fruta, *Grapholita molesta* (Busck) (Lepidoptera: Tortricidae) es una plaga importante en los cultivos de frutas de hueso. La hembra de *G. molesta* emite una mezcla de tres componentes de feromona compuesta de acetato de (*Z*) -8 dodecenilo (*Z*8-12: Ac), acetato de (*E*) -8 dodecenilo (*E*8-12: Ac), y alcohol de (*Z*)-8 dodecenilo (*Z*8-12:OH), en una proporción de 100:6:10, respectivamente. La actividad de las polillas macho se guía por estas moléculas en un ambiente de olores de planta. La mezcla de feromona sintética se utiliza en el manejo de plagas. Ambos sexos explotan los olores de plantas para encontrar su planta huésped y los machos para encontrar a sus congéneres. El objetivo principal de esta tesis es profundizar en la neuroetología olfativa de *G. molesta* a olores de feromonas y plantas huésped a través de estudios de comportamiento y fisiología.

Tres mezclas de plantas diferentes (Australiana, China y Suiza) fueron probadas en el túnel de viento y sinergizron la respuesta de los machosa una concentración de feromona subóptima poco concentrada. Además, la mezcla de feromona y olores de planta disminuyó el tiempo que tomó a los machos las respuestas de vuelo, en comparación con la feromona sola. Por el contrario, estas mezclas de planta disminuyeron capturas de feromonas en el campo, y lo hicieron de una manera dependiente de la dosis. Por otro lado, el estímulo de planta po sí solo, no atrajo a *G. molesta*, ni en el laboratorio ni en el campo. La atracción de los machos se redujo a unas dosis subóptimas de feromonas muy concentrada, y la combinación de esta dosis con la mezcla de plantas no mejoró las respuestas de vuelo de los machos. La proporción de acetato de dos componentes en la mezcla de feromona es crítico para la atracción de machos. Curiosamente, la mezcla de la planta mejoró la respuesta de los machos a una mezcla de feromonas que contieíae una proporción no natural de los dos acetatos.

El papel de *Z*8-12: OH en la mezcla de feromonas no es del todo claro. En mi tesis he realizado ensayos para probar el papel de *Z*8-12:OH y alcoholes relacionados, y su interacción con los volátiles de plantas. 12:OH (un ingrediente de la feromona de *G. molesta*) y *E*8,*E*10-12:OH (codlemona, la feromona sexual de *Cydia pomonella*) suplantaron el papel de *Z*8-12:OH cuando este fue retirado de la mezcla. Esto demuestra que varios alcoholes químicamente relacionadas pueden jugar el mismo papel que el alcohol de la feromona. Pero aún más interesante fue el hecho de que la mezcla planta también podría sustituir la ausencia del alcohol.

El sinergismo entre feromona y volátiles de planta muy probablemente se inicia a nivel del sistema nervioso central, pero algunos estudios en polillas muestran que

ya puede comenzar mediante interacciones entre feromonas y olores de planta que ocurren en las neurona olfativas receptoras de feromona (ph-ORN). Para explorar esta posibilidad primero caracterizé la morfología las sensilas de la antena con microscopía electrónica de barrido y después investigué la respuesta electrofisiológica de las neuronas olfativas del receptor de machos *G. molesta* mediante electrofisiología extracelular. El 72% de las sensilas eran de tipo trichodeo y alojaban ph-ORNs. Los principales componentes de la feromona, *Z*8-12:Ac y *E*8-12:Ac, fueron detectados por ORNs altamente especializados, y su proporción en las antenas (100: 11,6, respectivamente) fue similar a su proporción en la mezcla emitida por las hembras (100: 6, respectivamente). Ninguna ORN fué receptiva al componente minoritario (*Z*8-12:OH). La respuesta de las Z-ORNs fue muy específica, mientras que las las E-ORNs también respondieron al isómero Z, aunque con menor sensibilidad. Alrededor del 30% de las ORNs de las sensilas trichodeas no respondieron a ninguno de los componentes de feromonas analizadas, pero algunas de éstas células respondieron a volátiles de planta. Los olores de la planta fueron detectados por una clase diferente de neuronas olfativas que las de la feromona, con diferentes grados de especialización y se alojaban en sensilas auricillicas. La estimulación de Z ORNs con mezclas binarias de *Z*8-12: Ac y dosis biológicamente relevantes de olores de plantas en dosis crecientes disminuyó ligeramente su respuesta a la feromona sexual. La respuesta de E-ORNs a una combinación de E8-12: Ac y vegetales volátiles no fué diferente de la estimulación con *E*8-12: Ac solo. Estimulación con la mezcla de plantas solas no cambió la actividad electrofisiológica de las neuronas Z y E. Mi conclusión es que el efecto sinergista de feromona y volátiles de planta probablemente no tenga lugar en la ph-ORNs sino que podría ocurrir en las neuronas del lóbulo antenal, como en otras polillas. En conclusión, los resultados presentados en esta tesis permiten ampliar el conocimiento del comportamiento y los mecanismos neuronales de feromonas y olores de planta en la respusta de machos de *G. molesta*.

Palabras clave: *Grapholita molesta*, olfato, registro de sensila única, feromonas sexuales, volátiles de plantas, túnel de vuelo, electrofisiología.

GENERAL INTRODUCTION

General Introduction

The dissertation is based on four chapters as shown in the contents.

Oriental fruit moth, biology and control

The oriental fruit moth, *Grapholita molesta* (Busck), is a widely distributed tortricid fruit pest attacking stone (*Prunus* spp.) and pome (*Malus*, *Cydonia*, and *Pyrus* spp.) fruits in the regions situated between 20° and 60° latitude in both hemispheres. The center of origin of *G. molesta* is thought to be in Northwest China from where its current distribution has expanded through international trade and transport of fruit material (Rothschild and Vickers, 1991; Horak and Komai, 2006; Zheng et al., 2013). The incidence of fruit or shoot damage varies between 20 and 30%, and it can reach 80% when OFM populations are high. Depending on the region, *G. molesta* undergoes 3-6 generations per year. Females lay eggs singly on the lower surface of the leaves and near the growing shoot tips. First generation larvae bore into the tips of shoots and feed mainly on the xylem tissue. When mature, larvae leave the shoot to pupate inside a cocoon under bark or on the ground. Second generation larvae usually feed inside the shoot tips, but sometimes attack the ripening early peach fruits. Later generations may attack shoots, but the females prefer to lay eggs on or near ripening fruit. Larvae of fifth-sixth generations overwinter in the cocoon of the pupal stage. Small larvae usually enter the fruit near the stem end, or anywhere on the surface, especially where two fruits touch. Serious damage occurs when larvae bore the fruit and feed around the pit because this lowers fruit grade and causes serious economic damage to the growers. Feeding wounds can serve as infection sites for brown rot disease (Strand, 1999).

Currently pesticide applications are the main control strategy to keep the population below economic threshold level. Sprays are taken up specifically matching the time of newly hatched caterpillars before they bore into shoots or fruits. Although chemical control is an effective method, large-term use of chemical pesticides alone can increase the possibility of resistance development and pose serious threats to food safety related to human health (Usmani and Shearer, 2001; Kanga et al., 2003). After the identification of the female-produced sex pheromone of *G. molesta* (see below), insecticide sprays are timed with pest occurrence by monitoring moth activity with pheromone traps (McLaren et al., 1999). In addition, sex pheromone is employed in a large scale to reduce adult mating by the mating disruption technique (Witzgall et al., 2010). In most cases, mating disruption can eliminate the need for insecticide sprays (Strand, 1999). The rationale behind the large scale use of pheromone dispensers is to out-compete the tiny amounts of pheromone released by females (Fig. 1), which reduces the chances of males finding their mates (and so the name "mating disruption"). It is a promising and powerful tool for environmentally safe control and more than 50,000 ha of peach and apple orchards are treated with sex pheromones for mating disruption of *G. molesta* (Witzgall et al., 2010). Although, mating disruption is very successful in bringing down moth populations by affecting male perception, female behaviors do not seem to be affected. On the other hand, the use of mating disruption is restricted to low

population densities and to isolate orchards, where immigration of gravid females is precluded (Witzgall and Arn, 1997).

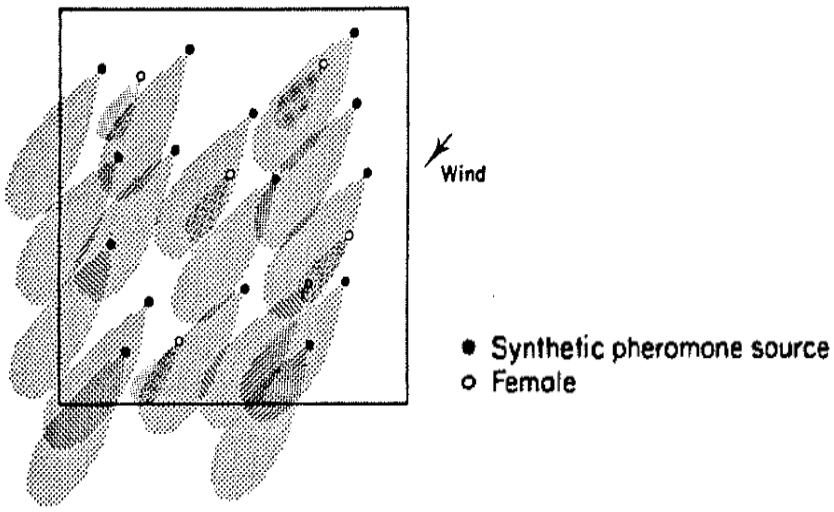

Figure. 1. Diagrammatic representation of how plumes of synthetic pheromones outcompeting calling virgin females (Birch and Haynes, 1988).

Odor guided behavior

The composition of a moth pheromone blend is species specific and guarantees the encountering of conspecific individuals. Female *G. molesta* release a chemical blend from their sex pheromone gland composed of *Z*-8-dodecenyl acetate (*Z*8-12:Ac), *E*-8-dodecenyl acetate (*E*8-12:Ac), and *Z*-8-dodecenol (*Z*8-12:OH), and a blend with a 100:6:10 ratio of these components, respectively, is most attractive to the males (Baker and Cardé, 1979; Cardé et al., 1979). A behavioral study shows that too low or too high doses of pheromone decrease male *G. molesta* responses (Varela et al., 2011a). In addition, slight changes in the ratio of pheromone components in a blend reduce male responses drastically (Knight et al., 2015). The role of *Z*8-12:OH in the pheromone blend is less clear (Han et al., 2001; Jung et al., 2013) and males accept wide variations of this compound (Linn and Roelofs, 1983; Linn et al., 1986). Female gland extraction studies identified another alcohol, 12:OH, shown to have a role in close range behavior (Carde et al., 1979). Pheromone components from other species can enhance or inhibit the behavioral responses of *G. molesta*. For example, a mixture of *G. molesta* pheromone and codlemone, the major sex pheromone component of *Cydia pomonella* L., increased trap captures of male *G. molesta*, while it decrease *C. pomonella* captures (Knight et al., 2014). The alcohol component of *G. molesta´*s sex pheromone, *Z*8-12:OH inhibits closely related species *Grapholita funebrana* (Treitschke) and *Grapholita prunivora* (Walsh), that use a similar ratio of the Z/E acetates (Guerin et al., 1986).

In addition to pheromones cues, males also use host plant cues to find females to mate, since females choose suitable host plants to lay eggs (Landolt and Phillips, 1997). Plants emit up to 10% of their assimilated carbon into the atmosphere as volatile organic compounds of which there are about 30,000 different molecules, including hydrocarbons,

alcohols, aldehydes, esters, carboxylic acids and terpenoids (Peñuelas and Llusià, 2004). Several studies aim to exploit the attraction of male and female moths to host plant volatiles (Bruce and Pickett, 2011) derived from a large variety of secondary metabolites (Pichersky and Gershenzon, 2002). Currently, efforts are dedicated to investigate the potential use of pheromones and other semiochemicals in pest management (Szendrei and Rodriguez-Saona, 2010). Similar to the pheromone blend composition, it is crucial for an insect to detect the right proportion of individual plant odors to find a right host and its habitat (Bruce and Pickett, 2011; Baker et al., 2012).

Compared with sex pheromones, there are relatively few examples of successful plant volatile lures for pest control (Szendrei and Rodriguez-Saona, 2010). One such example is the pear ester (ethyl-(*E*,*Z*)-2,4-decadieonate), a volatile released by ripe pear fruit that preferentially attracts the codling moth, *C. pomonella* (Knight et al., 2011).Studies report the attraction of both male and female *G. molesta* to plant odors in different locations. In Australia, a volatile blend emitted by young peach shoots (a 1:2:2 ratio of (*Z*)-3-hexenyl acetate:(*E*)-β-farnesene:(*E*)-β-ocimene, respectively) captured up to 130 males/trap, and (*E*)-β-farnesene and (*E*)-β-ocimene alone captured close to 600 males/trap (Il'ichev et al., 2009). In China, several synthetic volatile blends identified from fruits of different pear and peach varieties were tested in peach and pear fields and one of them (a 1:1:100:70:7:5:1:4 ratio of 1-hexanol:nonanal:ethyl butanoate: butyl acetate: ethyl hexanoate: hexyl acetate: hexyl butanoate: farnesene, respectively), captured about 50 males and 20 females per trap, which was just 5 times less than what commercial pheromone traps captured in the field (Lu et al., 2012; 2014). The same blend resulted in 10% source contact in the wind tunnel, equivalent to the response to the natural fruit (Lu et al., 2012). Analysis of peach shoot volatiles in Switzerland derived in a blend (a 100:20:3:20:0.5 ratio of (Z)-3-hexenyl acetate:(Z)-3-hexenol:(E)-2-hexenal:benzaldehyde:benzonitrile, respectively), which in dual-choice olfactometer tests was as attractive to mated females as the natural odor (Piñero and Dorn, 2007). The Swiss blend has not been tested under field conditions, and the Australian and Swiss blends have not been tested under laboratory conditions. In addition, these 3 blends are tested in different locations and in period, but not simultaneously in the same location.

There is evidence that the behavioral response of males to sex pheromone is increased by host plant volatiles (Reddy and Guerrero, 2004). The simultaneous presence of pheromone and plant odors could either help locating a mate, mask the female pheromone, or be neutral, without any effect on the female emitted pheromone (Deisig et al., 2014). The Swiss blend was tested with males in a wind tunnel and although it did not stimulate flight on its own, it synergized male response to a sub-optimal low pheromone dose (Varela et al., 2011a). Another tortricid moth study reports that plant volatiles enhance the decreased male response caused by high pheromone doses (Schmidt-Büsser et al., 2009). It is unknown how a plant blend will affect male *G. molesta* responses to an overdose pheromone blend. A correct ratio of *Z*- and *E*8-12:Ac compounds in the pheromone blend, that resembling the female gland release, is very critical for successful male *G. molesta* response (Knight et al., 2015). Plant blends synergize male responses to under-dose pheromone blend, however, the role of plant odors on male *G. molesta*

responses to unnatural pheromone blend compositions is unknown. The Australian and Chinese plant blends have not been tested for pheromone-plant synergism in either laboratory or field conditions. In addition, no study has tested if a plant blend can replace the role of *Z*8-12:OH in the two-acetate blend lacking Z8-12:OH. Therefore much remains to be done on the effect of plant volatiles on male moth response to suboptimal pheromone blends.

Mechanisms of odor processing

After the discovery of the first insect pheromone while studying insect responses to apparently invisible signals in *Bombyx mori* L., many studies were focused on describing chemical and behavioral roles, and understanding the mechanisms of pheromone reception (reviewed by Jacquin-Joly and Lucas, 2005). Some of the major questions of chemical ecologist's interests are: How are odors detected with such an amazing sensitivity and specificity, and are transuded at the peripheral level? How is the peripheral information represented in the higher brain centers? How is the entire odor mixture of an ecologically relevant situation represented? How is such a representation modified by experience? (Hansson, 2002).

Each animal has plenty of peripheral sensors that enable the detection of different sensory stimuli, including light, chemicals, sound and vibration, temperature, and humidity (Hansson, 1999). Multiple sensors present on the animal offer many functional advantages to perceive and respond to environmental signals. These advantages include extending the ability to detect and determine the spatial distribution of stimuli, improving the range and accuracy of discrimination among stimuli of different types and intensities, and increasing behavioral sensitivity to stimuli (Derby and Steullet, 2001). Insect antennae, equivalent to the nose of vertebrates, come in a wide variety of shapes and sizes, and is divided in to scape, pedicel and flagellum, with a few to multiple flagellomers (Keil, 1999).

Chemosensory neurons detect odors and are housed in a cuticular structure present on the antennae and other appendages, which are called sensilla. Different types of sensilla have been described: s. styloconica, s. chaetica, s. coeloconica, s. auricillica, s. basiconica and s. trichodea including their function (Schneider, 1964) (Fig.2). Olfactory neurons are housed in a sensilla located on the antennae, whereas gustatory receptor

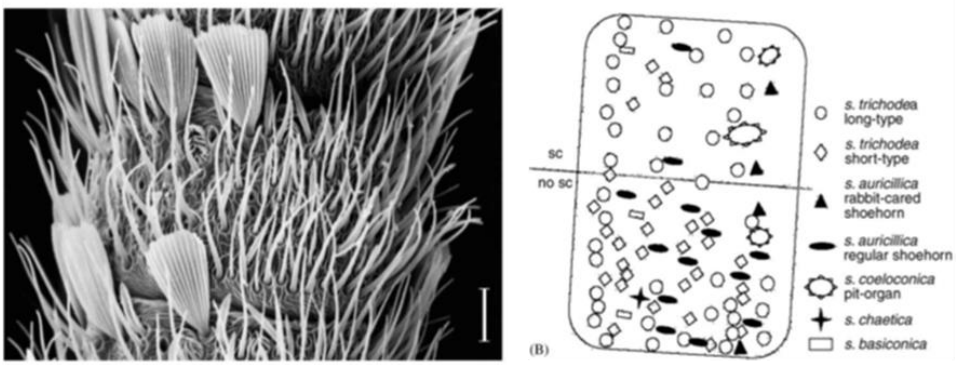

Figure 2. Distribution of sensilla types on the flagellum of male codling moth, *C. pomonella* (modified from Ansebo et al., 2005).

neurons are found on different parts of the body (like antennae, tarsi, mouthparts, ovipositor, and wings). George and Nagy (1984) described ultrastructure and different types of sensilla located on the *G. molesta* antennae, and suggest the need of electrophysiological studies to confirm some of their ultra-structure morphological results in sensilla trichodea. Abundance and distribution is known for sensilla trichodea and basiconica in *G. molesta*, but not for other sensillum types (George and Nagy, 1984). An olfactory sensillum consists either one or few to several bipolar olfactory sensory neurons (ORNs) surrounded by a special set of concentrically arranged auxiliary cells, forming cuticular, subcuticular and glial elements (Keil, 1999). The number of ORNs per sensillum varies depending on the species and their biological adaptations (Keil, 1999).

Studies in several moths show specific pheromone component detection by distinct ORNs, which may be housed singly in a sensillum trichodeum or together with other pheromone ORNs (reviewed by De Bruyne and Baker, 2008; Baker et al., 2012). It is evident that moths need a correct blend to identify their conspecifics. Interestingly, there is a correlation between the proportion of ORNs that respond to the major and minor pheromone components and the relative abundance of these compounds in the female-produced sex pheromone blend (Fig. 3) (Baker et al., 2012). In contrast to ph-ORNs (pheromone ORNs), ORNs tuned to plant volatiles are generalists, respond to several odorants with varied sensitivity (de Bruyne and Baker, 2008). Studies show that ORNs responding to plant odorants are as sensitive as ph-ORNs, and that plant ORNs are housed in different types of sensilla than ph-ORNs (Binyameen et al., 2012). In *G. molesta* single-sensillum recordings of ph-ORNs have been performed (Baker et al.1988; Hoskovec et al., 1996) but there are no studies specifically exploring the response profile of ORNs to pheromone components or plant odors.

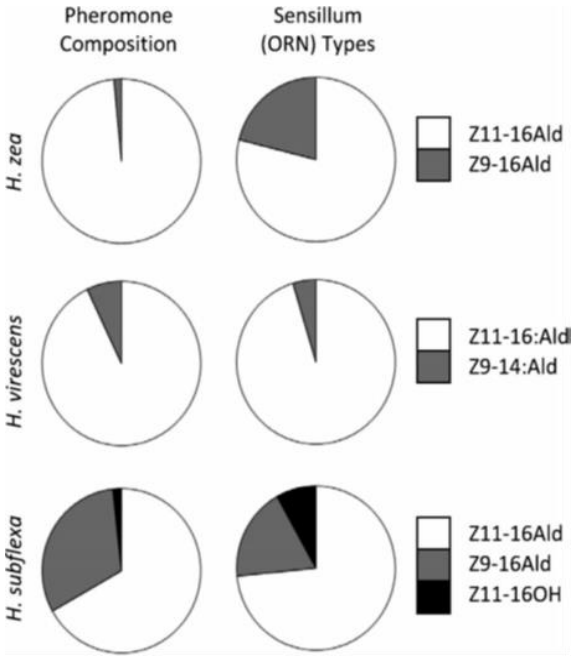

Figure 3. Percentage of sensillum types (right column) with ORNs tuned to the indicated sex pheromone components of 3 North American heliothine species, *Heliothis virescens*, *Heliothis subflexa*, and *Helicoverpa zea* (Baker et al., 2012).

The olfactory information collected by ORNs from the environment converges in the mid-section of the insect´s brain called deutocerebrum. The part of the deutocerebrum that receives all this antennal input is called the antennal lob (AL) (Hansson and Anton, 2000). The AL is organized in glomeruli, and each of them formed by the synapses of ORNs that share the same olfactory receptor protein with neurons that carry the information further down the nervous system. The arrangement and number of glomeruli within the AL are largely species specific, allowing the identification of individual glomeruli according to size, shape and position (Hansson and Anton, 2000). All synaptic interactions in the AL happen within the glomeruli, while its outer part is formed by axons and dendrites from the connecting neurons. The number of glomeruli is species specific and ranges from about 50 in Diptera, around 60 in moths, 160 in honeybees to more than 1000 in locusts (Anton and Homberg, 1999; Ignell et al., 2005; Rospars, 1988; Vosshall et al., 2000). A macroglomerular complex (MGC) located at the entrance of the AL exclusively receives input from ph-ORNs, whereas ordinary glomeruli (OG) receive input from plant-sensitive ORNs (Hansson and Anton, 2000; Lei and Vickers, 2008). Numerous OG on the AL allow insects to identify and quantify a vast number of non-pheromonal odorants (Anton and Hansson, 1995; Christenesen and Hildebrand, 2002; Diesig et al., 2014).

Several studies strive to understand the mechanism of behavioral pheromone-plant synergism in the brain (*i.e.* antennal lobe, AL) of insects (Deisig et al., 2014 and references there in). In most cases, integration of odors occurs in the AL (Hildebrand, 1995; Diesig et al., 2014). However there is evidence in some moths that plant odors interact at the antennal level, in the ph-ORNs. For example, in male *Heliothis zea* (Boddie), stimulation with a binary mixture of (*Z*)-11-hexadenal (the major pheromone component) and increasing doses of either linalool or (*Z*)-3-hexenyl acetate, significantly synergise ph- ORNs firing rate compared with responses to the major pheromone component alone (Ochieng et al., 2002). By contrast, inhibition was observed in ph-ORNs to the stimulation of pheromone and plant odors in *Heliothis virescens* (Fabricius) (Hillier and Vickers, 2011), *Spodoptera littoralis* (Boisduval) (Party et al., 2009) and *Agrotis ipsilon* (Hufnagel) (Deisig et al., 2012). So, the plant odors either synergize or inhibit ph-ORNs responses of pheromone components. However, there is no documentation of behavioral synergism or inhibition of pheromone and plant blend in male *G. molesta* at peripheral receptor neuron level.

This thesis is designed based on the following objectives to answer the gaps raised in the introduction. Preamble is laid out for each main and sub-objective, as follows.

OBJECTIVES

Objectives

Objective 1

Field studies in Australia and China report attractant blends (Australian, Chinese and Swiss). These blends have been tested at different locations, but it would be interesting to compare their attractancy when tested side-by-side. In addition a wind tunnel study (Varela et al., 2011a) has shown that the Swiss plant blend synergize male *G. molesta* responses to under-dosed sex pheromone. However the synergistic effect of the Australian and Chinese blends remains to be tested. Therefore the first objective of my thesis is:

a) To compare the attractancy of the three plant blends (Australian, Chinese and Swiss) simultaneously, and b) To determine if two plant blends (Australian and Chinese) synergize male G. molesta *responses to under-dosed pheromone.*

The attractancy of the three plant blends has been tested under different conditions. The Australian blend has been tested only in the field, the Swiss has been tested only in the laboratory and the Chinese blend in both field and laboratory conditions, but each blend has been tested independently of the others and there is no estimation of how attractive they are with respect to each other. So the first part of objective 1 is:
Objective 1.1: To compare the three plant blends side-by side and to do this under both laboratory and field conditions.

The synergism of the Swiss blend has been tested in the wind tunnel but not under laboratory conditions, whereas the synergism of the Australian and Chinese blends has not been tested under any conditions. So the second part of objective 1 is:
Objective 1.2: To determine the synergism of the three plant blends to an under-dose pheromone stimulus under both laboratory (wind tunnel) and field trapping conditions.

Objective 2

The synergism of plant blends with suboptimal low concentrations of sex pheromone has been demonstrated in *G. molesta* and other species. However very few insect studies have explored whether plant blends can synergize other forms of suboptimal sex pheromone, such as pheromone blends that are too high in concentration or pheromone blends lacking components or having unnatural ratios of their components. The second objective of my thesis is:

To determine the synergistic effect of plant blends a) on an over-concentrated pheromone dose and b).on unnatural configurations of the sex pheromone.

A dose-response curve (Varela et al., 2011a) has shown that plant volatiles are effective at increasing male response to a sub-optimal pheromone dose. In that same study it is reported that male response decreases at the highest pheromone doses. This first part of the second objective is:
Objective 2.1: To determine the effect of plant volatiles on the response to a suboptimally overdosed sex pheromone concentration.

Small alterations of the ratio of the two acetates (*Z*8-12:Ac and *E*8-12:Ac) in the pheromone blend of *G. molesta* relative to the optimal natural ratio result in strong reductions in male response (Knight et al., 2015), but whether plant volatiles can restore attraction to "off-blends" has not been tested in *G. molesta* or in any other moth species. Therefore the second part of the second objective is:

Objective 2.2: To determine if a plant blend can enhance male attraction to unnatural ratios of E8-12:Ac in the pheromone blend.

The third pheromone component in the blend of *G. molesta* is the alcohol *Z*8-12:OH. The role of this compound in the pheromone blend is not as clear as that of the acetates. Some studies show that this compound has a significant effect and other studies show that it is not necessary. Other studies show that additional alcohol compounds may be part of the pheromone blend, and yet other studies show that the pheromone of *C. pomonella*, which is a closely related alcohol compound, enhances the response of *G. molesta* to its own pheromone. To investigate the role of alcohols in the attraction of *G. molesta* to the sex pheromone I carried out two further objectives:

Objective 2.3.1: To determine if a plant blend can substitute the role of the alcohol Z8-12:OH in the response of males to a blend lacking this compound.

Objective 2.3.2: To determine if other alcohol components can replace the role of Z8-12:OH in a pheromone blend lacking this compound.

Objective 3

Previous studies show that plant blends synergized male *G. molesta* responses to suboptimal pheromone stimuli, however the physiological mechanisms responsible for this effect are not known. Plant blends and sex pheromone information probably integrate in the brain. However studies in other moth species reveal that the interaction of pheromone and plant studies starts in ph-ORNs. So my third objective is:

To determine if pheromone and plant odors interact in ph-ORNs of male G. molesta.

The ph-ORNs of *G. molesta have* not been characterized. It remains to be determined where these ORNs are located, whether the ORNs of each pheromone component occupy the same sensillum or occur in different sensilla, what is the sensitivity and specificity of these neurons and their abundance. The first part of this objective is:

Objective 3.1: To characterize the ph-ORNs of male G. molesta.

Because the effect of stimulus concentration is important in this kind of experiments, in order to determine behaviorally meaningful plant volatile doses that may represent real field conditions, the second part of this objective is:

Objective 3.2: To characterize the response of non-pheromone ORNs to plant odorants and construct dose-response curves.

Finally, after having characterized the ph-ORN types and having determined the meaningful doses of plant odorants, the last part of this objective is:

Objective 3.3: To determine if plant volatiles alter the response of ph-ORNs to pheromone.

CHAPTER I

Low reproducibility of attraction to plant lures in the moth *Grapholita molesta* (Lepidoptera: Tortricidae)

Low reproducibility of attraction to plant lures in the moth *Grapholita molesta* (Lepidoptera: Tortricidae)

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Abstract

Studies carried out in Australia, China, Spain and Switzerland have shown that several plant volatile blends attract *Grapholita molesta* (Busck) (Lepidoptera: Tortricidae), males and females or synergize male response to the sex pheromone. The goal of our study was to test these blends side by side, in the field and in the laboratory. Plant blends alone did not attract males or females in either field or laboratory settings, despite the use of different doses, dispensers and solvents. The only exception was a significantly larger number of males captured by β-ocimene than by solvent traps in the field (1.4 and 0.2 males/trap/check, respectively). Plant blends, instead, decreased pheromone captures in the field, and did so in a dose-dependent manner. By contrast, in the wind tunnel males responded better (higher number and faster responses) to mixtures of sex pheromone and each of the three plant volatile blends than to sex pheromone alone. We discuss the discrepancy between our results and former studies, as well as between laboratory and field tests.

Keywords: oriental fruit moth, volatiles, wind tunnel, traps

1. Introduction

The oriental fruit moth, *Grapholita molesta* (Busck) is a pest of peach and apple and is controlled with a combination of insecticide applications and mating disruption (Kong et al. 2014). With the ultimate goal of developing plant attractants for *G. molesta* males and females, several plant volatile blends have been identified from host-released volatiles and have been tested under laboratory and field conditions, obtaining responses that are comparable to the natural host. In Australia, a volatile blend emitted by young peach shoots (a 1:2:2 ratio of (*Z*)-3-hexenyl acetate:(*E*)-β-farnesene:(*E*)-β-ocimene, respectively) captured up to 130 males/trap, and (*E*)-β-farnesene and (*E*)-β-ocimene alone captured close to 600 males/trap (Il'ichev et al. 2009). In China, several synthetic volatile blends identified from fruits of different pear and peach varieties were tested and one of them (a 1:1:100:70:7:5:1:4 ratio of 1-hexanol:nonanal:ethyl butanoate:butyl acetate:ethyl hexanoate:hexyl acetate:hexyl butanoate:farnesene, respectively), captured about 50 males and 20 females per trap, which was just 5 times less than what commercial pheromone traps captured in the field (Lu et al. 2012; 2014). The same blend resulted in 10% source contact in the wind tunnel, equivalent to the response to the natural fruit (Lu et al. 2012). Analysis of peach shoot volatiles in Switzerland derived in a blend (a 100:20:3:20:0.5 ratio of (*Z*)-3-hexenyl acetate:(*Z*)-3-hexenol:(*E*)-2-hexenal: benzaldehyde:benzonitrile, respectively), which in dual-choice olfactometer tests was as attractive to mated females as the natural odor (Piñero and Dorn 2007). The Swiss blend was tested with males in a wind tunnel and although it did not stimulate flight on its own, it synergized male response to a suboptimal pheromone dose (Varela et al. 2011).

Our study has three aims, a) to determine the reproducibility of previous Australian and Chinese field studies, b) to compare side by side plant blends that have been tested independently (Australian and Chinese), and c) to test under field conditions a blend (Swiss) that has been tested only in the wind tunnel. To this end the "Australian", "Chinese" and "Swiss" blends, and a "Total" blend containing all the components from the other blends, were tested together in a heavily infested peach orchard in Chile, and the majority of the field treatments were further tested in the wind tunnel. In addition, two individual compounds (β-ocimene, and terpinyl acetate) which have shown behavioral activity in previous studies (Il'ichev et al. 2009; Knight et al. 2014) were tested in the field in Chile. Plant blends were tested alone or mixed with sex pheromone, at several doses and with different solvents and dispensers, to provide *G. molesta* with a wide range of stimulus concentrations in the air.

2. Materials and methods

2.1. Insects

The colony of *G. molesta* was maintained at the University of Lleida, Spain, since 2005 and originated from a laboratory rearing established at Piacenza, Italy, with insects collected from orchards in that locality. Larvae were reared on a semi-synthetic diet modified from Ivaldi-Sender (1974) under a L16:D8 photoregime at 25 ± 1° C. Pupae were separated by sex and were placed in 4-L polypropylene containers provided with a

cotton ball soaked in 10% sugar dissolved in water. Adults were collected daily and used when 2-4 days old.

2.2. Chemicals

Pheromone aliquots were prepared from a concentrated hexane solution of (*Z*)-8-dodecenyl acetate (*Z*8-12:Ac), *E*8-12:Ac, and (*Z*)-8-dodecenol (*Z*8-12:OH) (Pherobank, Wageningen, The Netherlands, > 99% pure) in a 100:5.4:10 ratio. Plant odorants were purchased from Sigma-Aldrich (Santiago, Chile, chemical purity, product and lot numbers in Table 1). The composition of the plant blends followed those reported by the Australian (Il'ichev et al. 2009), Chinese (Lu et al. 2012), and Swiss (Piñero and Dorn 2007) studies (Table 2). A fourth plant blend which contained all the pheromone components of the other three blends was included in the tests ("Total" blend, Table 2). Stock solutions were prepared from pure compounds and diluted in hexane or in mineral oil as needed.

2.3. Field tests

Fields tests were carried out in peach orchards in Chile in 2012-2013 (Duao, Maule, 35°33'29"S, 71°33'44"W) (Table S1). White-color delta traps (215 mm long x 200 mm wide x 100 mm tall) were used in all the experiments except for the pheromone treatment in experiment 1, where the traps were of red color due to a temporary shortage of white traps (Figure S1). Trap color does not affect *G. molesta* captures (Zhao et al. 2013). Traps were placed at eye level, hanging from 4-cm-diameter blue PVC pipes (Figure S1) fitted in the tree branches. Traps within a plot were placed in a transect 15-20 m apart, and plots were at least 15 m apart from each other. Trap floors were lined with removable sticky cards.

The lures were dissolved in hexane or in mineral oil and loaded in the large cup (500 μl capacity) of hexane-rinsed red sleeve-stopper rubber septa (3.4 x 6.6 mm bottom i.d. x o.d., Sigma Aldrich). Lures in experiment 1 were also dissolved in mineral oil and loaded in 1.5 ml eppendorf tubes with perforated (1.5-mm diameter) lids and fitted with a 15-mm-long x 7-mm-diameter section of dental cotton roll (Figure S1). The lid of the eppendorf tube was kept closed. In experiment 1 the dispensers were hung from the ceiling of the trap with a wire, almost touching the trap floor (Figure S1). In the other two experiments the dispensers were placed directly on the sticky surface of the trap floor. Septa and eppendorf tubes were labeled with the treatment name using permanent markers. Traps lured with pheromone and plant odors had 2 septa, one for each stimulus.Trap bottoms were replaced if there were captures. The sex of the captured individuals was determined in the laboratory under the stereo microscope.

2.3.1. Experiment 1

In this experiment we wanted to compare the attractiveness of the Australian, Chinese, and Swiss plant blends. A fourth blend ("Total") containing all the compounds from the other three plant blends was also included in the test (Table 2). We used two stimulus doses, high and low (Table S1), and two solvent types, hexane for rubber septa dispensers and mineral oil for eppendorf tube dispensers, with the purpose of providing a

broad range of volatile emission rates, although the release rates were not measured. Pheromone (80 μg) and solvent (hexane or mineral oil) were positive and negative controls, respectively, and were loaded in the corresponding dispensers (septum or eppendorf tubes). The 20 treatments [(4 plant treatments x 2 doses + solvent + pheromone) x 2 dispenser types (septum or eppendorf)] were placed in each of 4 rows, or plots. The experiment started in December 12, 2012 and ended in January 29, 2013. During this time there were 6 trap checks, so the number of experimental units (or sample size, N) was N=24 (4 plots x 6 checks). Pheromone and plant lures were loaded new in the first and second checks. For the remaining of the experiment the pheromone lure was unchanged and new plant lures were replaced one last time in the 4th check.

2.3.2. Experiment 2

Experiment 1 showed that the plant blends alone capture very few *G. molesta* males or females, so in this test we wanted to determine if the plant blends had any effect on the attractiveness of males to the sex pheromone. To this end we baited traps with a sex pheromone septum (16 μg) and added a second septum with the plant blends (using the "high" dose of experiment 1, Table S1). In this and the rest of the field tests, with one exception, we used only the rubber septum dispensers, not the eppendorf tubes. Pheromone-only traps served as positive controls and hexane and plant-only traps served as negative controls (same dose as in the pheromone-plus-plant treatment). Treatments were dissolved in hexane and loaded in rubber septa. The 10 treatments (4 plant blends; 4 pheromone-plant blends; solvent; pheromone) were replicated in 8 plots and run between January 29 and February 12, 2013, with 5 trap checks every 3 to 4 days, so the sample size was N=40 (8 plots x 5 checks). Pheromone septa were not replaced, and new plant lures were replaced a second and last time in the 3rd check.

2.3.3. Experiment 3

Experiment 2 confirmed the lack of attractiveness of the plant blends on their own, and showed that plant blends reduce captures in sex pheromone traps. In this new experiment we wanted to confirm the inhibitory effect of the plant blends observed in experiment 2 by testing the effect of three plant doses (low, medium and high) of the two plant blends that caused stronger inhibition in experiment 2 (Chinese and Total) (Table S1). In addition we wanted to determine if the inhibition caused by plant blends in experiment 2 could be related to the use of two septa in the pheromone-plus-plant treatments, as opposed to just one septum in the pheromone-only treatment. In here we compared a treatment that had just one pheromone septum with a treatment that had one pheromone septum and one hexane septum. Hexane septa were tested alone to control for possible pheromone contamination. All the hexane septa, whether tested alone or with sex pheromone, were prepared together. Plant blends alone were not tested in here due to their minimal effect in the previous tests, but two new plant odorants, terpinyl acetate and β-ocimene, were tested alone and in combination with sex pheromone (Table S1), as these compounds have shown behavioral activity in other studies (Il'ichev et al. 2009; Knight et al. 2014). The Australian blend (high dose) was tested in open eppendorf tubes with mineral oil because this treatment was relatively successful at capturing females in

experiment 2. Pheromone dose was 8 μg in all treatments. The 14 treatments (2 plant blends x 3 doses with pheromone; pheromone alone; pheromone and hexane; hexane; 2 plant odorants with and without pheromone; Australian blend) were placed in 4 plots and received 6 plant checks (every 1 to 2 days, it was population peak, February 12th to 25th, 2013 in Chile), so the sample size was N=24 (4 plots x 6 checks). Pheromone septa were not replaced, and new plant lures were replaced one last time in check 4.

2.4. Flight tunnel tests

The flight tunnel consisted of a 150 x 45 x 45 cm (length x height x width) glass cage with a solid white floor and a sliding door on one side. A 30-cm-diameter fan at the upwind end of the tunnel, and a 20-cm-diameter exhaust vent at the downwind end created a 0.35 m s^{-1} wind flow of unfiltered room air through the tunnel that was vented outside of the building after exiting the tunnel. Temperature inside of the tunnel was 23 ± 1°C. The flight tunnel was illuminated from above with fluorescent light bulbs producing 150 lux of white light. Tests were carried out during the last 3 hours of the photophase and occasionally into the first hour of the scotophase, in which case the daylight illumination was left on. Males were placed individually in 100 x 20 mm glass tubes with perforated aluminum lids covering both openings and were transferred to the flight tunnel room 30 to 120 min before the beginning of the test. Test odors were applied in 10 μl loads to 10 x 15 mm hexane-rinsed filter paper pieces (Whatman® No. 1, Sigma-Aldrich, Barcelona, Spain). The filter paper was held by a 30-mm alligator clip and was placed in a fume hood for 5–10 min to let dry before transferring to a 20 ml clean vial, where it remained until tested in the flight tunnel 5 to 180 min later. The glass vial containing the test odor was opened and closed inside the flight tunnel to minimize contamination of the flight tunnel room. The base of the alligator clip was inserted vertically in the slot of a 25-mm binder clip, itself fixed to a 70-mm diameter aluminum metal plate located on top of a 25-cm-tall metal-wire platform (0.5 cm mesh). The filter paper's flat surface faced the wind flow to attain a sufficiently turbulent odor plume. Three to five males were flown to each filter paper treatment before changing for another treatment paper. At the end of a test day a filter paper had been used with 8–15 males, so that filter papers were outside of the glass vial and exposed to the wind flow between of 32 to 60 min before being discarded. In a given day only one filter paper was used for each treatment. After placing the odor stimulus in the upwind platform the male cage was placed in the flight tunnel on top of a metal-wire platform similar to the one used for the odor source and 1.5m downwind from it. The aluminum lid was removed and we recorded if the male took flight, started upwind oriented flight (zig-zagging upwind flight) or landed on the filter paper containing the stimulus source, and the time it took him to engage in these behaviors. Each male was given 2 min to respond. At the end of the day the interior of the flight tunnel was cleaned with ethanol and the exhaust fan was left on. All glass and metal utensils were thoroughly rinsed in acetone and oven-dried at 200°C. Treatment order was randomized. The number of treatments was high (20) so they were tested in two groups on alternate days.

2.4.1. Experiment 4

The following treatments were tested in the wind tunnel: Australian, Chinese and Swiss plant blends at 10 μg each, a suboptimal sex pheromone dose of 1 ng (a response curve to doses of 1, 10, 100 and 1000 ng resulted in 34, 82, 89 and 63% source contact respectively, N=44, similar to Varela et al. 2011), and sex pheromone: plant blends (Australian, Chinese or Swiss) at 1:0, 1:10, 1:100, 1:1,000, and 1:10,000 ratios with pheromone at 1 ng. These were the doses present in the 10 μl sample volume loaded on the filter paper (Table S1). In addition 20 insects were tested to hexane on random days to control for contamination. The pheromone:plant blends were prepared using a stock pheromone solution so that all had identical pheromone concentration. The 1:0, 1:100, 1:1,000 and 1:10,000 blends were prepared on January 18, 2013 and the 1:10 blend on February 4, 2013. Wind tunnel tests were carried out between February 8th and 27th, 2013 with N=64.

2.5. Statistical analyses

A generalized linear model (GLM) with a Poisson family link in the package lme4 of R (R Development Core Team, 2015) was used to analyze trap count data (Bolker et al. 2009). Due to the high temporal variation in trap captures, sampling date was included as a random effect in the model (GLMM), and so was the variation among plots if they contributed significantly to the model after comparing among models with ANOVA. The percentage of males responding in the wind tunnel was analyzed with GLM models using a binomial family link. Behavioral categories (take flight, oriented flight and contact) were analyzed separately. Treatments with no responding insects were added one responding individual in a randomly chosen replicate so that the percentage of response was > 0 and the GLM model could converge. The time to respond in the wind tunnel was analyzed with a linear model and the data were transformed [log (x+1)] to approach a normal distribution. Response times were not analyzed if there were no or very few responders. Comparisons among treatment pairs in both field and wind tunnel studies were performed with the glht or lsmeans functions of R using Tukey´s alpha correction method. The data shown in the figures corresponds with the predictions from the models. Raw data and R codes (with selected statistical outputs, including P-values) are provided as supplementary files. Whenever the term "significant" is used in the text it means that the significance level is $P \leq 0.05$.

3. Results

3.1. Experiment 1

Only one female was captured in the entire experiment, in a trap baited with a high dose of the Swiss blend diluted in mineral oil loaded in an eppendorf tube. The 4 pheromone septa traps captured a total of 1,632 males, and the 4 pheromone eppendorf traps captured a total of 215 males. All the plant volatile traps combined, which summed 64, captured a total of 64 males in the entire sampling period. Of the 64 males collected by the plant volatile traps, 61 were captured in a single sampling week (week 4), and these

captures were clustered mostly in 3 single traps: 35 males in a single trap baited with low-dose Swiss-blend loaded in a septum, 15 males in a single trap baited with a high-dose Chinese-blend loaded in an eppendorf tube, and 9 males in a single trap baited with a high-dose Total blend loaded in a septum. This level of captures in plant-baited traps was not observed before or after week 4 (only 3 more males where captured in the rest of the experiment in plant-baited traps) or in experiment 2, so we suspect that this is the result of an isolated pheromone contamination event, probably resulting from the use of pheromone-contaminated rubber gloves. Table 3 summarizes total trap captures. Due to the low number of insects captured, no statistical analysis was performed on these data.

A large number of small flies (up to 111 per trap, Table 3) were captured in traps baited with plant volatiles. Morphologically the flies could be separated into what appear to be three different species (Figure S2) most likely belonging to the families Chloropidae and Milichiidae (Irina Brake, personal communication). The response of these flies was blend- and dose-specific because they were found mainly in the Chinese and Total traps and in higher numbers in the high than in the low dose traps. As with the response of *G. molesta* to sex pheromone, more flies were attracted to septa than to eppendorf tubes. A preliminary fly exclusion test indicated that the presence of flies in the traps did not affect moth captures (data not shown).

3.2. Experiment 2

Plant blend or hexane traps captured no males on their own, whereas pheromone traps captured 2,800 males in total (Figure 1). Only 9 females were captured in this experiment, but all of them in the same treatment: pheromone with the Australian plant blend. The addition of a plant blend septum of any of the plant blends to a trap baited with a pheromone septum significantly decreased the number of males captured by pheromone. This negative effect was significantly stronger for the Chinese and Total blends than for the Australian and Swiss blends.

3.3. Experiment 3

A total of 13,650 males and 17 females were captured in this experiment. Six females were found in pheromone traps baited with β-ocimene and 4 in pheromone traps baited with terpinyl acetate, but traps baited with pheromone and the Australian blend captured no females. Significantly fewer males were captured in pheromone traps with the Chinese and Total blends than in pheromone-alone traps, and this effect was significantly stronger as the plant blend dose increased (Figure 2). Pheromone traps with two septa, one for pheromone and another for hexane, captured significantly more males than traps having just one pheromone septum, whereas traps with just the hexane septum captured no males (Figure 2). β-ocimene captured significantly more males than hexane traps, but far fewer males than pheromone traps (Figure 2). Terpinyl acetate captured no moths on its own but significantly increased the capture of males by pheromone (Figure 2).

3.4. Experiments 4

Several doses of the Australian, Chinese and Swiss blends were added to sex pheromone and the percentages and speed of responses to the pheromone-plus-plant blends was then compared with that of pheromone-only lures. None of the plant blends alone, or hexane, attracted any males at the single dose tested (10 μg), so they were not included in the mean comparison test. Pairwise comparisons between each pheromone:plant treatment and the pheromone alone treatment showed that the three plant blends significantly increased the percentages of flight, oriented flight and contact, and did so in a dose-dependent manner (Figure 3A). The Chinese and Swiss blends significantly increased responses at the 1:100 to 1:1,000 pheromone:plant ratios, whereas the Australian blend did so at the 1:1 and 1:10 ratios. The three plant blends significantly reduced the time of response to the sex pheromone, and, as with the percentages of response, the effect was stronger at the higher (plantwise) pheromone:plant ratios (1:1,000 and 1:10,000) (Figure 3B).

4. Discussion

4.1. Response to plant blends

Our study shows that the plant blends tested are not attractive to *G. molesta* males on their own, neither in field trap tests, nor in laboratory flight tunnel tests. This contrasts with previous studies which show moderate to high responses of *G. molesta* males to the same plant volatile stimuli that we have tested. The previously untested "Total" blend, which was used only in the field and contained all the odorants from the other three blends, also failed to attract any males, so different combinations of similar chemicals did not make a more attractive blend. The only plant stimulus that caught any significant number of males in our field tests was β-ocimene, which at a 3 mg dose in rubber septum captured more males than the solvent traps that served as controls (1.5 and 0.2 males/trap/check, respectively). However, this level of captures is still far from the numbers captured in pheromone traps (66 males/trap/check). In the Australian study a 1 mg dose of (*E*)-β-farnesene or (*E*)-β-ocimene captured between 500 and 600 males/trap (Il'Ichev et al. 2009), far more than what we captured in our field test with a 3 mg dose of these compounds.

Although the plant blends were extremely poor attractants of *G. molesta* moths, they captured very large numbers of small flies, probably belonging to the families Chloropidae and Milichiidae, which are reportedly attracted to similar plant chemical stimuli (Zhang and Aldrich 2004). Capture of flies in our traps allows us to make several inferences regarding the properties of the plant stimuli tested. First of all, it shows that our test traps released plant odors that mimic natural blends that are of interest to some insects. Second, fly captures were affected by volatile dose and dispenser type (rubber septum versus eppendorf tube), which shows that the release rate of the volatiles varied with dose and dispenser, as it was intended. Third, the attraction of flies was blend-specific (each fly species preferred a different blend, data not shown), which shows that each plant blend released a different odor bouquet, as it was expected. Indeed, we could

distinguish the 3 plant blends and their concentration with our own sense of smell. Based on these observation we must conclude that although abundant *G. molesta* populations were present in the field tests, as indicated by pheromone trap captures, and that a relatively wide range of plant volatile blends and doses were released into the environment by the traps, by judging from the fly captures, the negligible number of male and female *G. molesta* captured reflects not a deficiency of the volatile stimuli tested, but a lack of attraction of this species to a relatively wide range of qualitatively and quantitatively diverse plant stimuli.

4.2. Effect of plant blends on sex pheromone response

Despite the poor response of *G. molesta* males and females to plant stimuli in our study, the same plant stimuli had a significant effect on the response of males to sex pheromone, and this effect went in opposite directions in laboratory and field settings, being synergistic in the wind tunnel and inhibitory in the field. Even the lowest pheromone:plant ratio (around 1:200) caused a significant inhibition in the field. In the wind tunnel the effect of the plant blends was just the opposite, they caused synergism both in percentage of males responding and speed of response, and it was stronger as the quantity of plant odor in the blend increased. So the plant blends have clearly significant and opposing effects on the response of *G. molesta* males to pheromone in laboratory and field settings. A recent field study with *G. molesta* shows that two plant volatiles [(*Z*)-3-hexenyl acetate and undecanol] synergize sex pheromone at a 1:0.5 pheromone:plant ratio, but that at the 1:1 and 1:2 ratios the synergism disappears, with a clear trend to become inhibitory at higher ratios (Yu et al. 2014). Therefore, the lowest pheromone:plant ratio that we used in our field tests was perhaps too high in plant odor to result in synergism. At a 1:10 pheromone:plant ratio, green leaf volatiles synergize the attraction of *Cydia pomonella* (L.), *Heliothis zea* (Boddie) and *Heliothis virescens* (F.) to sex pheromone (Dickens et al. 1993; Light et al. 1993), but at higher ratios, in the order of 1:100 and 1:1,000, green leaf volatiles also synergize the response of *Plutella xyllostella* (L.) (Dai et al. 2008). Clearly, the ratio between pheromone and plant stimuli appears to be a critical aspect to take into consideration in pheromone-plant studies because insects could be attracted or repelled by the same plant volatile depending on the dose.

4.3. Disagreement among studies

Disagreement in plant volatile responses between laboratory and field tests, or among studies, is not infrequent. The attraction of male *P. xylostella* to traps baited with sex pheromone in Canada is not affected when the leaf volatile (*Z*)-3-hexenyl acetate is added to the pheromone (Miluch et al. 2014). However in China and India, (*Z*)-3-hexenyl acetate synergizes the response of *P. xylostella* to sex pheromone (Reddy and Guerrero 2000; Dai et al. 2008). The flower volatile phenylacetaldehyde is a generalist noctuid moth attractant (Tóth et al. 2010) that increases the response of male *Spodoptera frugiperda* Walker to pheromone in the wind tunnel (Meagher and Mitchel 1998), however it decreases captures in pheromone traps in the field (Meagher 2001). Furthermore, the pear ester, although being an attractant for *C. pomonella*, performs very differently in different crops and locations (Knight 2010; El-Sayed et al. 2013; Tóth et al.

2014). There is increasing realization that background odors in the environment influence the response of insects to pheromone and plant stimuli (Bruce et al. 2005; Reinecke and Hilker 2014; Knudsen and Tasin 2015) and this could explain the disagreements among studies. Knudsen et al. (2008) show that the compound that attracts the apple fruit moth, *Argyresthia conjugella* Zeller, in the wind tunnel is different than the one that attracts it in the field, albeit both are released by the host, so the authors propose that the interaction of the plant volatiles with the background odor contributes to the different effect in field and laboratory.

Population differences in attraction to host volatiles could be important but they are rarely reported. The tephritid fly, *Rhagoletis pomonella* (Walsh) is a significant example of intraspecific differences in host plant preference. During the short time since the introduction of *Malus domestica* Borkh to North America, its natural habitat, it has undergone physiological and behavioral changes resulting in a new a race that prefers the newly introduced host, apple, over the natural host, hawthorn (*Crataegus douglasii* Lindl.) (Linn et al. 2012; Powell et al. 2014). We have reported lack of geographic variation of pheromone production and response in *G. molesta* (Knight et al., 2015), but whether population differences in host plant attraction occurs in this species remains to be tested. Field tests like those performed in Chile were carried out in Spain and indicate that this population is not attracted to the Swiss blend or to the pear ester in the field or in the wind tunnel, although here too the Swiss blend synergized pheromone in the wind tunnel (Figs. S3, S4, S5, S6), so the few data available for this species suggests that population differences are not a major factor in this case. This may be explained by the relatively recent human-aided expansion of a moth that shows reduced dispersal power (Wei et al. 2015).

Olfactory signal quality is yet another factor that could explain discrepancies among studies because releasers, solvents, doses and chemical compounds may vary among studies and affect what the insect antennae senses (Valeur et al. 1999; Tomaszewska et al. 2005). We have been careful to mimic the concentrations, releasers and solvents used in the Australian and Chinese studies, however none of the studies, including ours, has measured release rates and we cannot discard that differences in release rates varied among studies. Regarding the quality of compounds, in the Australian study the (*E*) isomers of β-farnesene and β-ocimene were used, whereas we used commercial racemic mixture of the (*E*) and (*Z*) isomers of both chemicals. Insects are known to distinguish structurally related plant isomers by means of isomer-specific olfactory receptor neurons (De Bruyne and Baker 2008), so discrepancy between the Australian study and ours could be explained by the racemic purity of the chemicals used in each case. Another difference between studies is that in China they used rubber septa in the wind tunnel whereas we used filter paper dispensers, which could have affected volatility (Valeur et al. 1999).

The unexpected higher captures of *G. molesta* in traps baited with two septa, one loaded with pheromone and another with hexane, than in traps baited with just one pheromone septum suggests that visual cues may play a role in the final steps that lead a male to be captured in these traps (Charlton and Cardé 1990; Rojas and Wyatt 1999;

Kuenen and Gilbert 2014). The Australian study used funnel-type traps designed to capture flies and this factor may account for the high *G. molesta* captures in that study (Il'ichev et al. 2009). However, captures were relatively high in our conventional delta traps baited with sex pheromone, so additional factors may explain differences among studies.

4.4. Concluding remarks

Poor moth response to synthetic plant volatile lures is not surprising given the difficulty to produce artificial lures that can compete with natural host blends under a background of volatile signals in the wild (Knudsen and Tasin 2015). Therefore, when a study reports levels of attraction with plant blends that approach those of the sex pheromone, one would expect that these blends would perform relatively well under a variety of experimental conditions. In the case of *G. molesta* we have found that relatively successful blends performed very poorly in our experiments, though we reproduced the experimental conditions of the original studies. Because our tests were not exhaustive, a number of uncontrolled variables could be responsible for the differences that we, and others, have encountered when trying to reproduce plant blends studies. These factors, mainly the characteristics of the stimulus itself (dispenser, concentration, chemical purity, trap type), the genetic architecture of the population, and the composition of background odors, should be taken into consideration because all of them have been shown to play a role in shaping insect response to plant volatiles.

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Table 1. Synthetic plant odorants used in the experiments.

Compound	CAS	Product number (Sigma Aldrich)	Lot number	Purity[a] (≥ %)
1-Hexanol	111-27-3	H13303	STBC8538V	98
Nonanal	124-19-6	W278203	STBC3506V	95
Ethyl butanoate	104-54-4	E15701	STBB7416V	99
Butyl acetate	123-86-4	402842	SHBB8826V	99.5
Ethyl hexanoate	123-66-0	148962	S28172V	99
Hexyl acetate	142-92-7	108154	STBC6608V	99
Hexyl butanoate	2639-63-6	W256803	STBC0651V	98
Farnesene (racemic)	NA	W383902	MKBG4494V	NA
(*Z*)-3-Hexenyl acetate	3681-71-8	W317101	MKBG6087V	98
(*Z*)-3-Hexenol	928-96-1	W256307	MKBG7249V	98
(*E*)-2-Hexenal	6728-26-3	W256005	STBC8608V	95
Benzaldehyde	100-52-7	B1334	STBC6885V	99
Benzonitrile	100-47-0	12722	BCBH8265V	98
β-Ocimene (racemic)	13877-91-3	W353901	MKBK5322V	90
Pear ester	3025-30-7	W314803	STBC4363V	80
Terpinyl acetate	80-26-2			95

[a] Sigma-Aldrich label

Table 2. Proportion of the odorants in each of the four plant-volatile blends. Actual quantities used in the tests are shown in Table S1.

Plant compound	Blend name			
	Australian[a]	Chinese[b]	Swiss[c]	Total
1-Hexanol		1		1
Nonanal		1		1
Ethyl butanoate		100		1
Butyl acetate		70		1
Ethyl hexanoate		7		1
Hexyl acetate		5		1
Hexyl butanoate		1		1
Farnesene[1]	100	4		1
(*Z*)-3-Hexenyl acetate	50		100	1
(*Z*)-3-Hexenol			20	1
(*E*)-2-Hexenal			3	1
Benzaldehyde			20	1
Benzonitrile			0.5	1
β-Ocimene[2]	100			1

[1] The Australian study used (*E*)-β-farnesene and the Chinese study used racemic farnesene. We used racemic farnesese

[2] The Australian study used (*E*)-β-ocimene, and we used racemic β-ocimene

[a] Il'ichev et al., 2009

[b] Lu et al., 2012 (JM blend, Table 2)

[c] Piñero and Dorn, 2007

Table 3. Captures of *G. molesta* males and females, and flies in Chile (experiment 1) in traps baited with one of 4 plant blends (Australian, Chinese, Swiss and Total) either dissolved in hexane and loaded in red rubber septa (top) or dissolved in mineral oil and loaded in eppendorf tubes (bottom). Two plant bled doses were tested (high and low). Solvent (hexane or mineral oil) and sex pheromone are negative and positive controls, respectively. Mean males/trap/check (SEM). A total of 1,911 males were captured, 64 in plant traps and the rest in pheromone traps. Only 1 female was captured. OFM captures were so low in the plant-baited traps that no statistical analysis was performed.

Septum										
	Pheromone 80 µg		Low dose (14-19 mg)				High dose (140-189 mg)			
Stimulus	Hexane	Pheromone	Australian	Chinese	Swiss	Total	Australian	Chinese	Swiss	Total
OFM males	0	68.00 (13.99)	0	0.08 (0.08)	1.46 (1.46)	0	0	0.04 (0.04)	0	0.38 (0.38)
OFM females	0	0	0	0	0	0	0	0	0	0
Flies	0	0	0	0	0	1.41 (0.84)	1.00 (0.98)	28.41 (8.25)	0.08 (0.09)	111.00 (33.13)
Eppendorf										
			Low dose (14-19 mg)				High dose (140-189 mg)			
Stimulus	Mineral oil	Pheromone	Australian	Chinese	Swiss	Total	Australian	Chinese	Swiss	Total
OFM males	0	8.96 (3.26)	0	0	0.04 (0.04)	0	0	0.62 (0.62)	0	0.04 (0.04)
OFM females	0	0	0	0	0	0	0	0	0.04 (0.04)	0
Flies	0	0	0	0.17 (0.13)	0	0	0	19.45 (5.30)	0	6.00 (3.27)

Table S1. Field experiment details (Chile)

Exp.	Objective	Start-end dates	Pheromone	Pheromone:plant ratio
1	Do plant blends attract OFM? Compare plant blends alone, at two doses and in two dispenser types (mineral oil in eppendorf tube vs hexane in rubber septum)	December 21, 2012 - January 29, 2013	80µg	(Plant blends tested alone) Australian: 100 mg (high)/10 mg (low) Chinese: 189 mg/19 mg Swiss: 143 mg/14 mg Total: 140 mg/14 mg
2	Is there pheromone-plant synergism? Compare pheromone-plant mixtures at one plant dose.	January 29 - February 12, 2013	16µg	Pher.:Australian, 1: 6,250 Pher.:Chinese, 1: 11,812 Pher.:Swiss, 1: 9,062 Pher.:Total, 1: 8,750 (Plant alone same as high conc. of exp. 1)
3	Is there pheromone-plant inhibition? Is inhibition dose-dependent? Compare pheromone-plant mixtures at several plant doses. Test additional plant compounds	February 12 - 25, 2013	8µg	Pher.:Chinese, 1:237.5 (low), 1:7,875 (medium), 1:24,750 (high) Pher.:Total, 1:175 (low), 1:5,825 (medium), 1:17,500 (high) Pher.:Ocimene/Terpinyl acetate, 1:375
4	Test blends of field experiments 1 to 3 in the wind tunnel	February 8 - 27, 2013	1ng	Pher.:Australian/Chinese/Swiss, 1:10 , 1:100, 1:1,100, 1:10,000 (Plant alone: 10 µg)

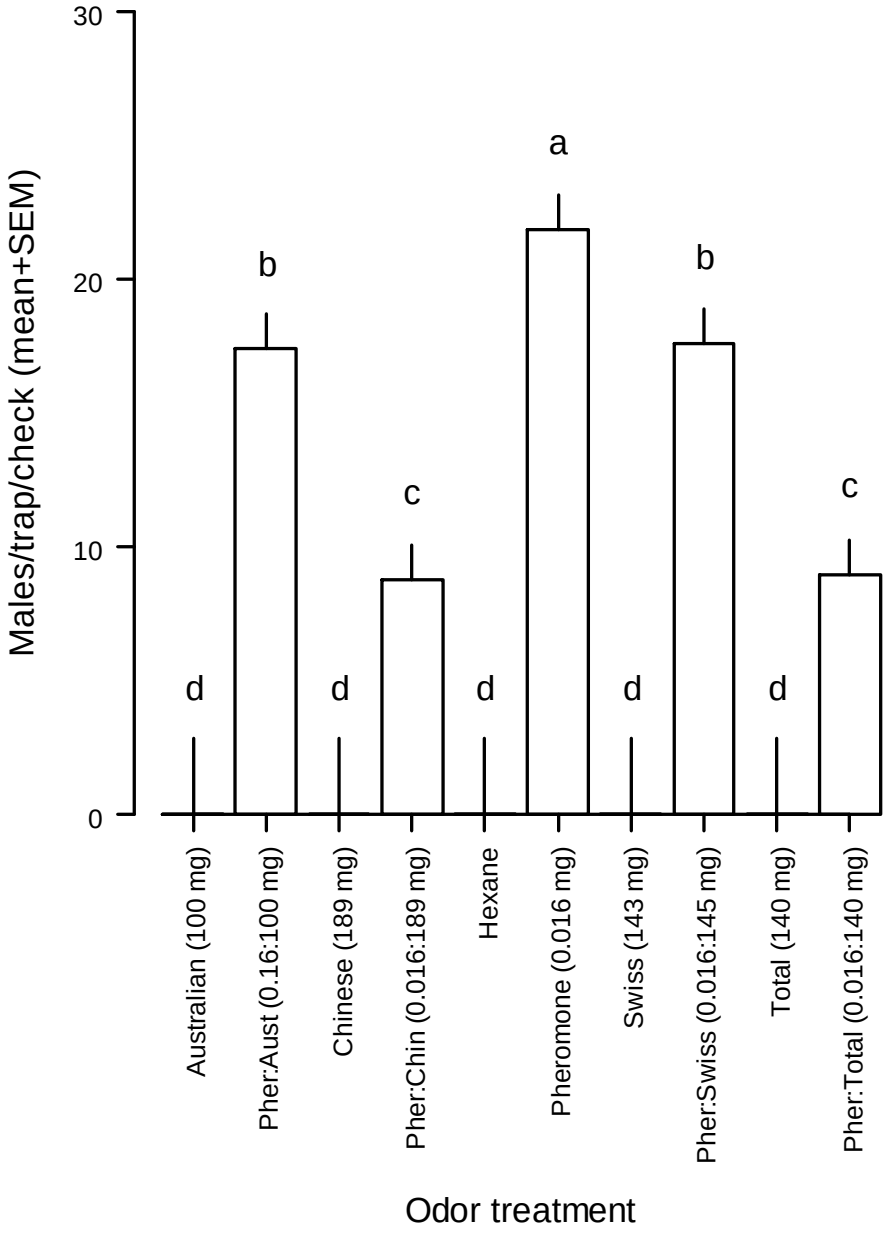

Figure 1. Effect of plant blends on the capture of *G. molesta* males in sex pheromone traps in Chile (experiment 2). Traps baited with either a) one dispenser loaded with one of the three plant blends (Australian, Chinese, Swiss or Total), sex pheromone, or hexane, or b) two dispensers, one with pheromone and another with a plant blend. Data shown in here are the predicted captures (mean and error) from the estimated parameters of a GLMM model. Letters indicate significant differences among treatment means following multiple pairwise comparisons using Tukey's test ($P < 0.05$). Plant blends significantly decreased the capture of males respect to pheromone-alone traps.

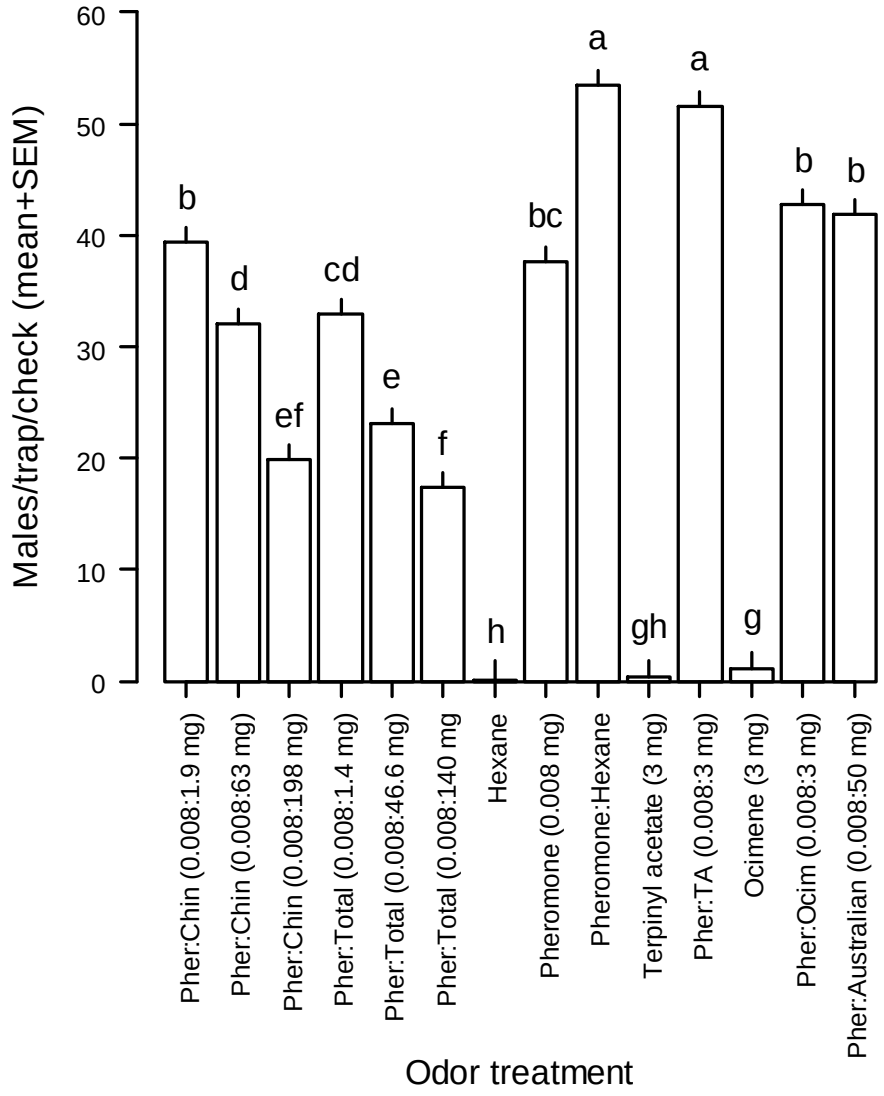

Figure 2. Effect of dose of Chinese and Total plant blends on the capture of *G. molesta* males in sex pheromone traps in Chile (experiment 3). Traps baited with either, a) one septum of either, sex pheromone, terpinyl acetate, β-ocimene or hexane, or b) a sex pheromone septum plus a second septum of each plant stimulus or hexane. Data shown in here are the predicted captures (mean and error) from the estimated parameters of a GLMM model. Letters indicate significant differences among treatment means following multiple pairwise comparisons using Tukey´s test ($P < 0.05$). All the plant treatments, except terpinyl acetate (TA) or hexane, decreased the capture of males in pheromone traps, and this effect was dose-dependent.

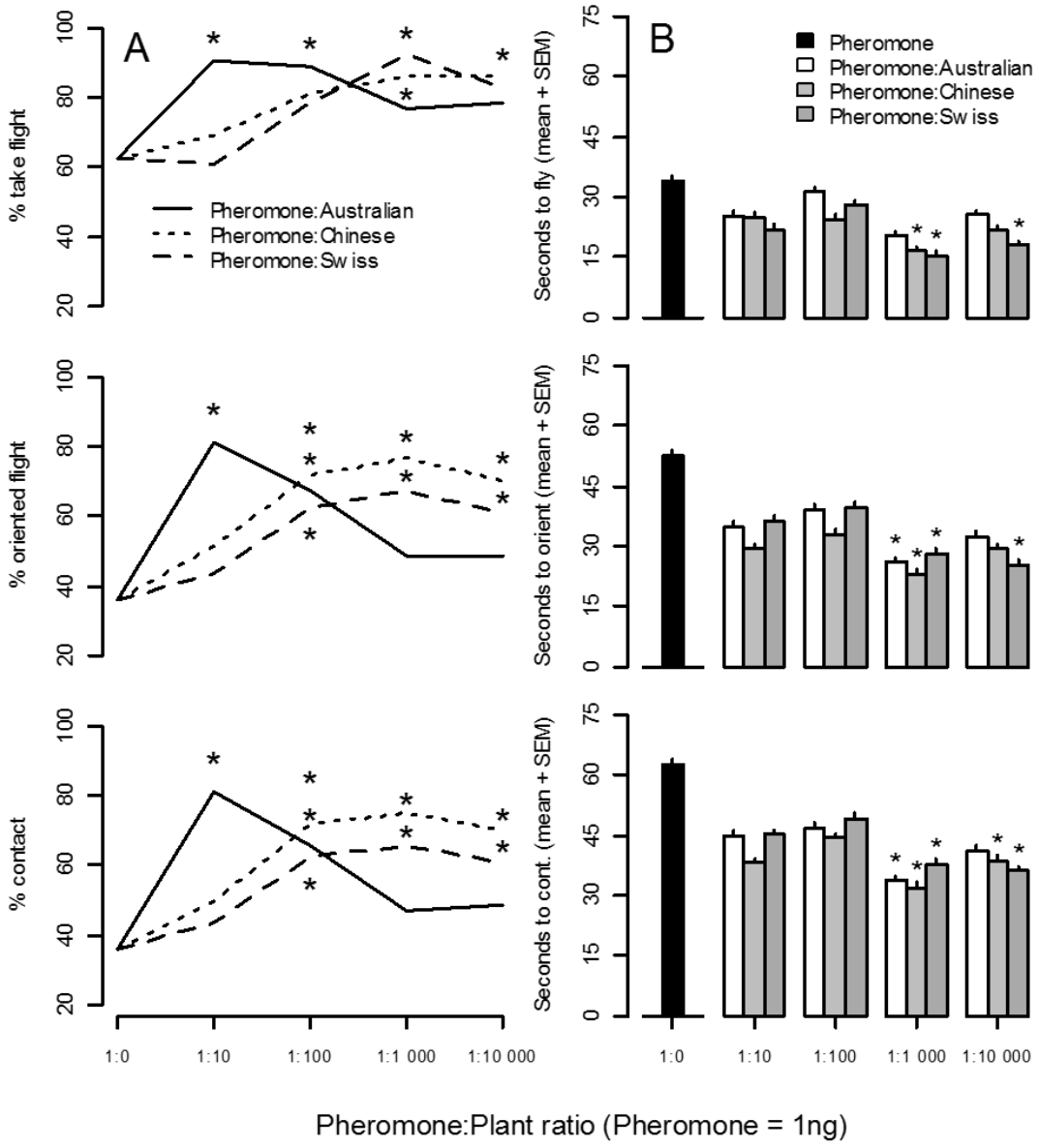

Figure 3. Effect of plant blend dose (Australian, Chinese and Swiss) on the response of *G. molesta* males to a suboptimal dose of sex pheromone in a flight tunnel (experiment 4). A) Percentage of males responding (take flight, oriented flight and contact). B) Time it took males to engage in these behaviors. Data shown in here are the predicted responses from the estimated parameters of GLM or LM models. Asterisks indicate significant differences between the pheromone-plus-plant treatments and the pheromone-alone treatment (1:0) by means of planned pairwise comparisons using Tukey`s test ($P < 0.05$)

Figure S1. Traps and liners, experimens 1, 2 and 3. White delta trap hanging from blue-PVC pipe holder (top left). Red delta trap showing hanging septum and sticky floor liner (experiment 1) (bottom left). Eppendorf tube with cotton roll (top center). Eppendorf tube lid perforated (bottom center). Eppendorf tube hanging above the sticky floor liner (experiment 1) (top right). Rubber septum hanging above the sticky floor liner (experiment 1) (bottom right).

Figure S2. Flies captured in plant volatile-baited traps experiments 1, 2 and 3. Sticky floor liner covered with hundreds of flies (top left). Flies are characterized by a sclerotized geniculate proboscis (top right). Flies could be separated in 3 species (sp1, sp2 and sp3) according to some morphological characteristics such as the size and shape of the proboscis, the color of the leg segments, and head protuberances (bottom).

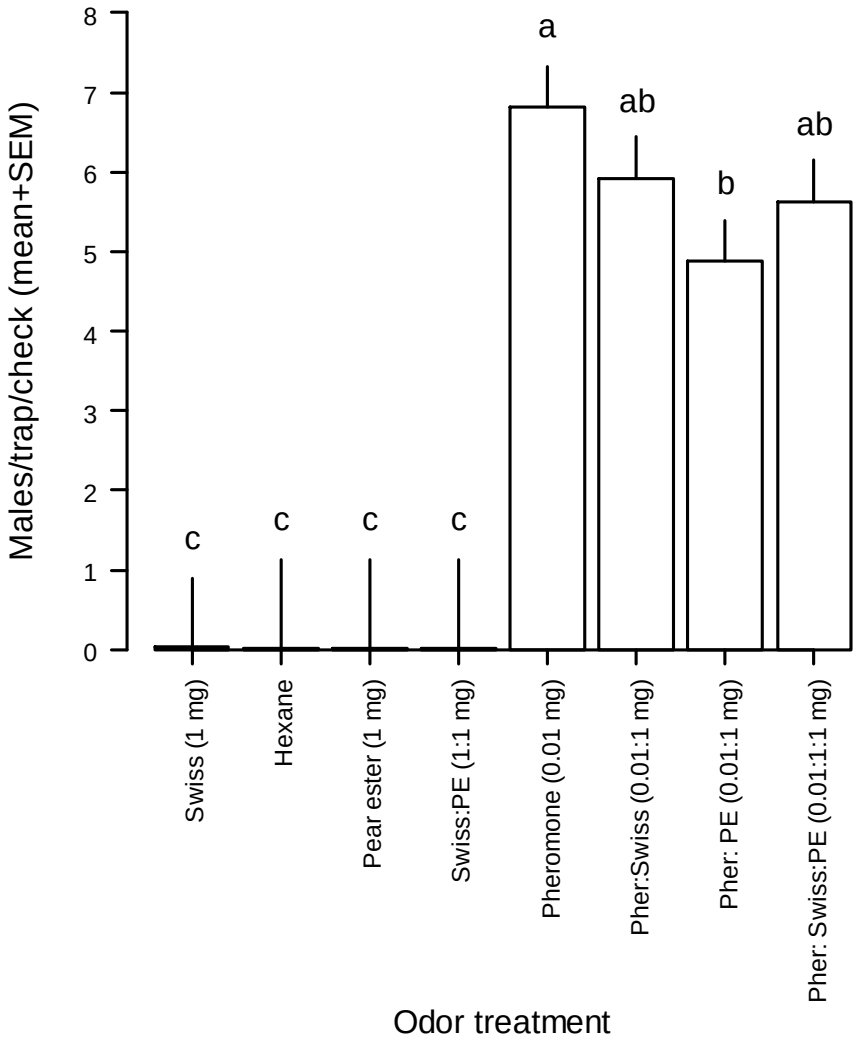

Figure S3. Captures of *G. molesta* males in delta traps in Spain in 2011 (La Portella, Lleida, 41°44′28″N, 0°38′30″E, July 5 to August 30). White delta traps were baited with the Swiss blend (Swiss), pear ester (PE), sex pheromone (Pher) and binary or ternary mixtures of the three compounds, one red rubber septum per trap loaded with the stimulus. There were 4 plots and 8 weekly checks (N=32) and septa were replaced on each check. Captures were analyzed with a general linear model (GLM) using a Poisson distribution. Data shown in here are the predicted mean captures and errors. Significant differences among treatments indicated with different letters (Tukey´s tet, $P < 0.05$).

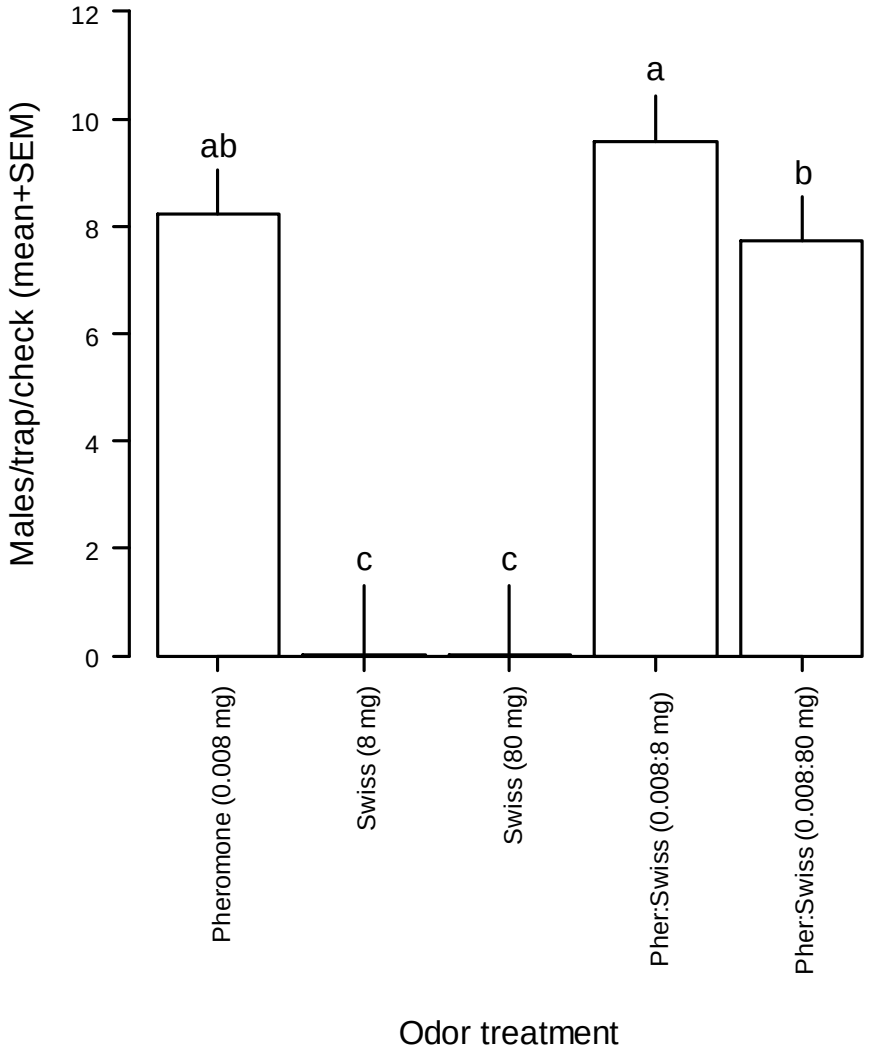

Figure S4. Captures of *G. molesta* males in delta traps in Spain in 2012 (Torrelameu, Lleida, 41°42′24″N 0°42′11″E, September 8 to October 11). White delta traps were baited with the Swiss blend (Swiss), sex pheromone (Pher) and binary mixtures of the two, one red rubber septum per trap loaded with the stimulus. There were 5 plots and 5 weekly checks (N=25) and septa were replaced on each check. Captures were analyzed with a general linear model (GLM) using a Poisson distribution. Data shown in here are the predicted mean captures and errors. Significant differences among treatments indicated with different letters (Tukey´s tet, $P < 0.05$).

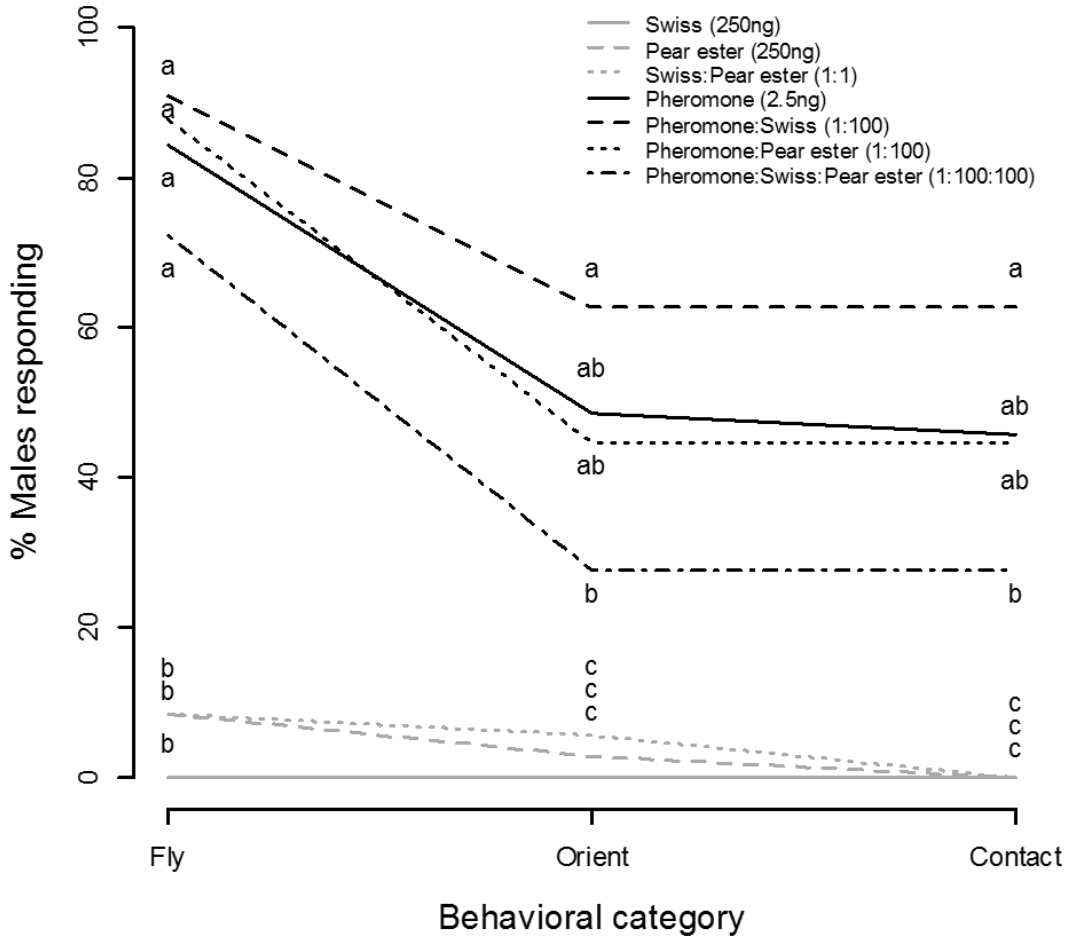

Figure S5. Effect of the Swiss blend and pear ester on the response of *G. molesta* males to sex pheromone in a flight tunnel (pheromone:plant ratio 1:100, experiment 7). Data shown in here are the predicted responses from the estimated parameters of a GLM model with binomial distribution. Different letters indicate significant differences among treatments within each behavioral category (take flight, orient and contact) using Tukey´s test ($P < 0.05$).

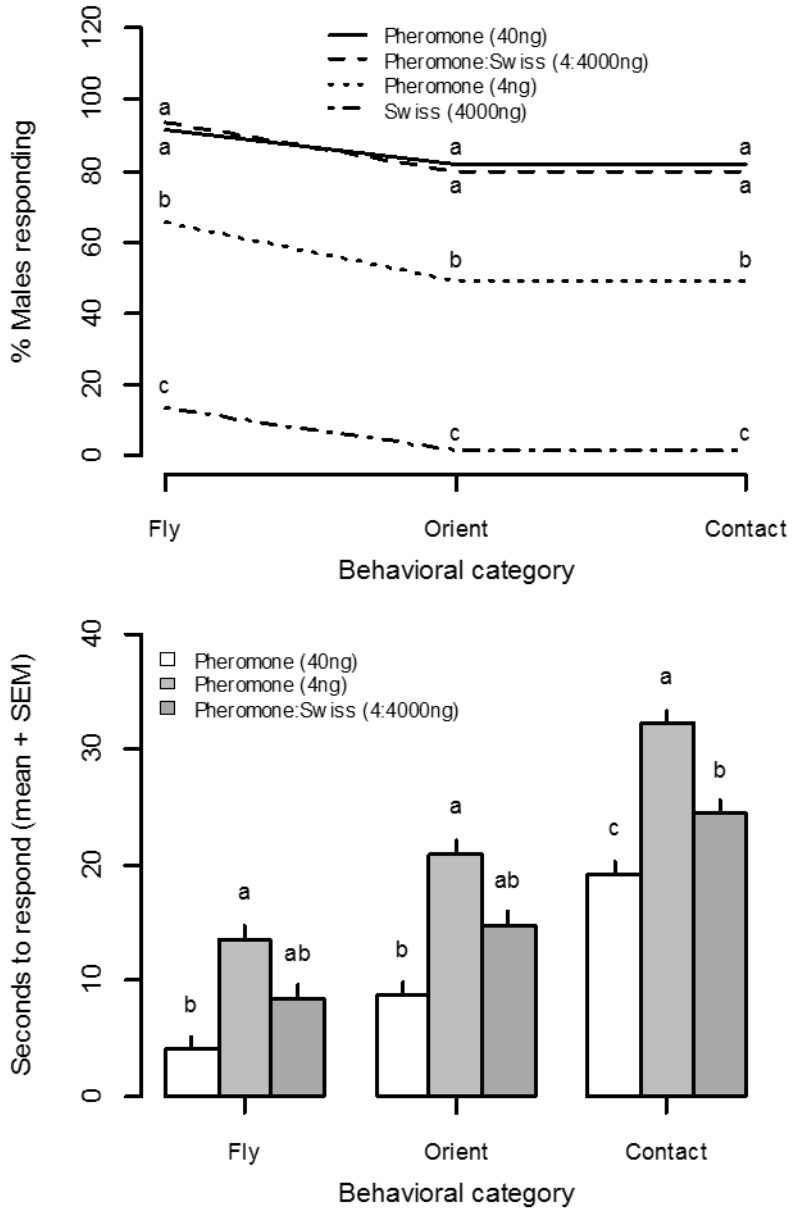

Figure S6. Effect of the Swiss blend on the response of *G. molesta* males to sex pheromone in a flight tunnel (pheromone:plant ratios 1:1,000 and 1:10,000). Top: Percentage of males responding (take flight, oriented flight and contact). Bottom: Time it took males to engage in these behaviors. Data shown in here are the predicted responses from the estimated parameters of GLM (percentage of response, binomial distribution) and LM (time to respond) models. Different letters indicate significant differences among treatments for each behavioral category (take flight, orient and contact) using Tukey´s test ($P < 0.05$).

CHAPTER II

Role of alcohols and plant volatiles on the response of male *Grapholita molesta* (Lepidoptera: Tortricidae) to sex pheromone in the wind tunnel

Role of alcohols and plant volatiles on the response of male *Grapholita molesta* (Lepidoptera: Tortricidae) to sex pheromone in the wind tunnel

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Abstract

Female *Grapholita molesta* (Busck) release a pheromone blend composed of two stereoisomeric acetates (*Z*8-12:Ac and *E*8-12:Ac) which must be present in an optimal 100:6 ratio in order to stimulate conspecific male approach. Plant volatiles have been shown to synergize male response to low pheromone concentrations in *G. molesta* and other moth species. In the present study we show that, in the wind tunnel, a host-derived plant blend also synergizes male response to pheromone blends containing unnatural E:Z ratios. The plant blend, however, did not synergize abnormally high pheromone doses. Departures from the optimal pheromone component ratio, or too high or low concentrations, result in fewer males responding to the pheromone. The alcohol *Z*8-12:OH is described as a third pheromone component because it significantly contributes to the attraction to the acetate blend, however its role as a natural part of the blend is not completely clear. We revisited the role of alcohols in the pheromone blend of *G. molesta* by testing the response of males to an optimal Z:E blend in which the natural alcohol, *Z*8-12:OH, was substituted by other alcohols. One of these, the sex pheromone of *Cydia pomonella*, *E*8,*E*10-12:OH, did supplant the role of the natural alcohol of *G. molesta*, *Z*8-12:OH, and so did the plant volatile blend, and both of them at the same ratio at which the natural alcohol synergizes the acetates (10%). Dodecenol (12:OH) which has been described as a fourth pheromone component of *G. molesta*, also increased responses but not as much as codlemone or the plant blend. Our results reveal new functions for plant volatiles on moth sex pheromone response under laboratory conditions, and shed new light on the role of alcohols ingredients in the pheromone blend of *G. molesta*.

Keywords: Insect, moth, olfaction, behavior

Manuscript

1. Introduction

Plants emit up to 10% of their assimilated carbon into the atmosphere as volatile organic compounds of which there are about 30,000 different molecules, including hydrocarbons, alcohols, aldehydes, esters, carboxylic acids and terpenoids (Peñuelas and Llusià, 2004). Plant volatiles are exploited by plant users, such as phytophagous insects, for the location and selection of their plants (Bruce et al., 2005; Szendrei and Rodriguez-Saona, 2010). Insects produce their own volatile signals, pheromones, to communicate with each other, and these are released into the environment and mix with the plant volatiles (Reinecke and Hilker, 2014). Both, plant volatiles and insect pheromones are species-specific blends of individual odorants, and although different plant species, as well as different insect species, share similar volatile molecules, the species-specific blend is the information that insects use to discriminate among hosts plants and conspecific species (Clifford and Riffell, 2013). The specificity of insect pheromones, and the strong responses that they elicit on insects, has made them a cornerstone tool in pest management practices, especially for the hundreds of moths species which sex pheromone has been identified and is used in mating disruption and population monitoring (Witzgall et al., 2010). However, pheromone detection occurs almost always against a background of plant odors, and there is growing evidence that this background can alter pheromone perception (Reinecke and Hilker, 2014).

Compared with sex pheromones, there are relatively few examples of successful plant volatile lures for pest control (Szendrei and Rodriguez-Saona, 2010). One such example is the pear ester (ethyl-(*E*,*Z*)-2,4-decadieonate), a volatile released by ripe pear fruit that preferentially attracts the codling moth, *Cydia pomonella* (L.) (Knight et al., 2011). Under mating disruption conditions fewer males are captured in sex pheromone traps and plant volatiles alone or combined with the sex pheromone may be more efficient attractants than sex pheromone alone (Knight et al., 2014). In addition, plant volatiles synergize insect response to pheromone (Reddy and Guerrero, 2004), and have the advantage of attracting both sexes, whereas pheromone traps will only attract males. However, for reasons still unclear, even successful plant attractants, like the pear ester, tend to perform erratically when compared with sex pheromones (Szendrei and Rodriguez-Saona, 2010; El-Sayed et al., 2013; Tóth et al., 2014).

The oriental fruit moth, *Grapholita molesta* (Busck) is a serious pest of pear, peach, apple, quince, and other stone fruits where the larvae bore into the young productive shoots or fruit and cause economical damage (Rothschild and Vickers, 1991). Female *G. molesta* emit a three-component pheromone blend composed of (Z)-8 dodecenyl acetate (*Z*8-12:Ac), (*E*)-8 dodecenyl acetate (*E*8-12:Ac), and (*Z*)-8 dodecenyl alcohol (*Z*8-12:OH) in a 100:6:10 ratio, respectively (Roelofs et al., 1969; Linn and Roelofs, 1983; Kong et al., 2014). Departures from the optimal ratio, or too high or low concentrations, results in fewer males approaching the source (Varela et al., 2011, Knight et al., 2015). Synthetic sex pheromone is used for monitoring and controlling the *G. molesta* by means of mating disruption (Witzgall et al., 2010; Kong et al., 2014). Behavioral and field trapping tests, together with chemical analysis of pheromone gland extracts and volatile collections, show that the two acetates are essential in pheromone

attraction, whereas the role of alcohol, Z8-12:OH, appears to be less crucial (Knight et al., 2015 and references therein). Furthermore, olfactory receptor neurons (ORNs) on the male antenna specific to each of the two acetates have been identified, whereas the antenna apparently lacks alcohol-specific ORNs (Ammagarahalli and Gemeno, 2014). Additional alcohols have been described as having effects on the pheromone response of *G. molesta* males. Dodecenol (12:OH), a component identified in pheromone gland extracts and volatile collections, affects the behavior of males when they are close to the pheromone source (Cardé et al., 1975a, b; Cardé et al., 1979). Interestingly codlemone, the alcohol pheromone component of *Cydia pomonella* L. [(*E,E*)8,10-12:OH] increases *G. molesta* male captures when mixed with the 3-component pheromone blend (Evenden and McLaughin, 2005; Knight et al., 2014). These observations prompted us to reinvestigate the role of alcohols in the pheromone system of *G. molesta*.

Analysis of peach shoot volatiles identified a plant blend composed of (*Z*)-3-hexenyl acetate:(*Z*)-3-hexenol:(*E*)-2-hexenal: benzaldehyde:benzonitrile in a 100:20:3:20:0.5 ratio, respectively, which in dual-choice olfactometer tests was as attractive to mated females as the natural host (Piñero and Dorn, 2007). This blend was tested with males in a wind tunnel and although it did not stimulate flight on its own, it synergized male response to a suboptimal pheromone dose (Varela et al., 2011). Synergism of plant volatiles on male moth pheromone response has been demonstrated for optimal or below optimal pheromone concentrations (Deisig et al., 2014, and references therein), but it has rarely been tested with above-optimal pheromone doses (Schmidt-Büsser et al., 2009). In this study we tested if plant volatiles could restore male response to suboptimally high pheromone doses. In addition we asked if plant volatiles, could increase male responses to pheromone blends having unnatural ratios of the Z/E acetate stereoisomers. Finally, we asked if the absence of *Z*8-12:OH from the pheromone blend could be replaced by other alcohols, or by the plant blend.

2. Materials and methods

2.1. Insects

The colony of *G. molesta* originated from a laboratory rearing established at Piacenza, Italy, with insects collected from peach orchards in that locality, and was maintained at the University of Lleida, Spain, since 2005. Larvae were reared on a semi-synthetic diet modified from Ivaldi-Sender (1974) under a L16:D8 photoregime at 25 ± 1º C. Pupae were separated by sex and were placed in 4-L polypropylene containers provided with a cotton ball soaked in 10% sugar water. Adults were separated daily and used when 2-4 days old.

2.2. Chemicals

Sex pheromone components of *G. molesta*, *Z*8-12:Ac, *E*8-12:Ac, *Z*8-12:OH, 12:OH and of *C. pomonella* major pheromone component, *E*8,*E*10-12:OH (codlemone), were purchased from Pherobank (Wageningen, The Netherlands) and they were shown to be >99% pure by GC-FID. Plant odorants were purchased from Sigma-Aldrich (Madrid, Spain, chemical purity, product and lot numbers in Table 1). A stock solution of

*Z*8-12:Ac, *E*8-12:Ac, and *Z*8-12:OH in a 100:6:10 ratio, respectively, was prepared from pure compounds, and dilutions were made in *n*-hexane as needed. The plant blend was prepared from pure compounds diluted in hexane with the same composition as reported by Varela et al. (2011) (Table 1), and it was diluted in *n*-hexane as needed. Further pheromone and pheromone:plant blends are described for each experiment.

2.3. Flight tunnel

The flight tunnel consisted of a 150 x 45 x 45 cm (length x height x width) glass cage with a solid white floor and a sliding door on one side. A 30-cm-diameter fan at the upwind end of the tunnel, and a 20-cm-diameter exhaust vent at the downwind end created a 0.35 m s^{-1} wind flow of unfiltered room air through the tunnel that was vented outside of the building after exiting the tunnel. Temperature inside of the tunnel was 23 ± 1°C. The flight tunnel was illuminated from above with fluorescent light bulbs producing 150 lux of white light. Tests were carried out during the last 3 hours of the photophase and occasionally into the first hour of the scotophase, but in this case the daylight illumination was left on. Males were placed individually in 100 x 20 mm glass tubes with perforated aluminum lids covering both openings. They were transferred to the flight tunnel room 30 to 60 min before the beginning of the test. Test odors were applied in 10 µl loads to 10 x 15 mm hexane-rinsed filter paper pieces (Whatman® No. 1, Sigma-Aldrich, Barcelona, Spain). The filter paper was held by a 30-mm alligator clip and was placed in a fume hood for 5-10 min to let dry before transferring to a 20 ml clean vial, where it remained until tested in the flight tunnel 5-180 min later. The glass vial containing the test odor was opened and closed inside the flight tunnel to minimize contamination of the flight tunnel room. The base of the alligator clip was inserted vertically in the slot of a 25-mm binder clip, itself fixed to a 70-mm diameter aluminum metal plate located on top of a 25-cm-tall metal-wire platform (0.5-cm-mesh). The filter paper's flat surface faced the wind flow to attain a sufficiently turbulent odor plume. Four to six males were flown to each filter paper treatment before changing the paper for another treatment. At the end of a test day a filter paper had been used with 8-10 males, so that filter papers were outside of the glass vial and exposed to the wind flow for a maximum of 30 min before being discarded. In a given day only one filter paper was used for each treatment. After placing the odor stimulus in the upwind platform the male cage was placed in the flight tunnel on top of a metal-wire platform similar to the one used for the odor source and 1.5m downwind from it. The aluminum lids were opened and recorded if the male took flight, started upwind oriented flight (zig-zagging upwind flight) or landed on the filter paper containing the stimulus source. Each male was given 2 min to respond. At the end of the day the interior of the flight tunnel was cleaned with ethanol and the exhaust fan was left on. All glass and metal utensils were thoroughly rinsed in acetone and oven-dried at 200 °C. Treatment order was randomized. The number of treatments was so high (24 and 23, experiment 2 and 3, respectively) that it was necessary to test them on alternate days or on morning and afternoon of same day.

2.4. Effect of plant volatiles on the response to overdosed pheromone blends

From the response of males to 0.1ng to 3µg pheromone doses a 2µg concentration was chosen as the overdose treatment to be used in this experiment. The overdose pheromone was mixed with several doses of the plant blend at 1:0.0001 to 1:100 pheromone: plant ratios, and these treatments were tested in the wind tunnel together with the optimal pheromone concentration (100ng), the overdose pheromone (2 µg) and the plant volatile alone (10 µg). In this experiment, in addition to counting the number of males flying, orienting and contacting the pheromone source, we also recorded whether the oriented males showed "arrested" flight, which is atypical behavior displayed by male moths when they are exposed to high pheromone concentrations, and which consists on the male stopping for a few second in mid-air at some distance from the odor source after having performed oriented flight.

2.5. Effect of plant volatiles on the response to pheromone blends with suboptimal Z/E isomer ratios

A stock solution with a 100:10 ratio (100:10 ng) of *Z*8-12:Ac and *Z*8-12:OH , respectively, was mixed with varying ratios of *E*8-12:Ac to make 0%, 50%, 100%, 160% and 200 % E-blends (percentage is with respect to the major pheromone component). Plant blend was added to these pheromone blends in ratios of 1:0, 1:1, 1:10 and 1:100 pheromone:plant. As a control we tested the optimal *E*8-12:Ac ratio (6%). In addition we tested an underdosed 6% E blend (1 ng) and the underdosed blend with plant volatiles (1:1000 ratio, respectively) to check the attractiveness of the plant blend as determined in a previous study (Varela et al., 2011).

2.6. Effect of alcohols and plant volatiles on the response to a pheromone blend lacking Z8-12:OH

A stock solution with a 100:6 ratio (100:6 ng) of *Z*8-12:Ac and *E*8-12:Ac, respectively, was mixed with varying ratios of *Z*8-12:OH, 12:OH, codlemone or the plant blend to make blends with a constant quantity of Z/E and 0%, 3%, 10%, 20%, 50% and 100% ratios of the alcohols, and plant blend (1:0.1 to 1:1000, pheromone: plant blend ratio, respectively) with respect to the major compound. The individual components of the plant blend were tested individually with the Z/E blend using the same amount as when they were in the blend.

2.7. Statistical analyses

A generalized linear model (GLM) with a binomial family link in the package lme4 of R (R Development Core Team, 2015) was used to analyze the percentage of males responding in the wind tunnel. Behavioral categories (take flight, oriented flight, contact and arrested flight) were analyzed separately. Treatments with no responding insects were added one responding individual in a randomly chosen replicate so that the percentage of response was > 0 and the GLM model could converge. Response times were not analyzed if there were no or very few responders. Comparisons among treatment pairs were performed with the glht functions of R using Tukey´s alpha correction method. The data shown in the figures corresponds with the predictions from the model. Raw data

and R codes (with selected statistical outputs, including models and pair-wise tests with their respective P-values, and tables with the observed data and the predicted values from the models) are provided as supplementary files. Whenever the term "significant" is used in the text it means that the significance level is $P \leq 0.05$.

3. Results

3.1. Effect of plant volatiles on the response to overdosed pheromone blends

There was a gradual raise in the behavioral response of males to increasing amounts of pheromone from 0.001 to 0.01 µg (Fig.1). As the concentration increased further a progressively higher percentage of orienting males arrested close to the source, resulting in 30% contacts with 2 µg and almost no contacts with 3 µg. For the following test the 2 µg concentration was chosen for the overdose treatment.

Plant blend alone stimulated 17% of the males to fly, but none oriented or contacted. 85% of the males oriented to the overdosed pheromone, but many also arrested and so there was only a 27% contact, significantly less than to the optimal which had 87% contacts and no arrested flights (Fig. 2). Addition of varying ratios of 5-component plant blend to the overdosed pheromone did not reduce the number of arrested flights, and so it did not increase the number of contacts and did not help the overdosed pheromone (Fig. 2).

3.2. Effect of plant volatiles on the response to pheromone blends with suboptimal Z/E isomer ratios

Unnaturally high or low ratios of *E*8-12:Ac resulted in significantly lower percentages of response, at all behavioral categories, than with the optimal 6% E-isomer ratio (Fig. 3). Addition of the plant blend to the unnatural E-ratio blends increased the number of flights to the 50%, 150% and 200% E-blends, and the number of oriented flights to the 150% E-blend (Fig. 3). All these synergistic effects were observed only at the 1:10 pheromone:plant ratio, but not at lower or higher ratios. A trend for increased contacts was observed but these differences were not statistically significant. Neither hexane nor the plant blend alone attracted any males. The plant blend significantly increased responses to an underdosed pheromone blend, confirming its synergistic effect (data not shown).

3.3. Effect of alcohols and plant volatiles on the response to a pheromone blend lacking Z8-12:OH

The addition of *Z*8-12:OH, 12:OH, codlemone and plant odors synergized male responses to an optimal Z/E blend that lacked alcohol, but the effect depended on the compound and concentration used (Fig. 4). *Z*8-12:OH synergized responses at the 10% dose only, codlemone synergized flight at 10% and 20% doses and oriented flight and contact at the 10% dose, and the plant blend synergized all behavioral steps at the 0.1% dose. Many other treatments increased male responses to levels not significantly different to the optimal blend, but in these treatments the response was not significantly different from the blend lacking alcohol either, so synergistic effect was weaker than in the

previous treatments (e.g., Z8-12:OH at 20, 50 and 100%, all the 12:OH doses except 10% contact and 100% oriented, Fig. 4). Finally, some treatments did not have any positive or negative effect on male response (e.g., Z8-12:OH 3% orient and contact, 12:OH 10% contact and 100% oriented, Fig. 4).

Because the synergistic effect of the plant blend occurred only at the lowest (0.1%) dose, we tested lower plant blend doses. In addition, because the plant blend is composed of several chemicals and one of them is an alcohol (Z3-6:OH), we further explored the role of each plant bled ingredient on pheromone-plant synergism. Here, as in the previous test, the 0% *Z*8-12:OH blend performed worse than the optimal 10% *Z*8-12OH blend, and the plant blend synergized at the 0.1% dose. Male responses to the 0.01% plant dose were not different from the optimal 10% Z8-12:OH blend but they were not different from the 0% Z8-12:OH blend either, so there was a weaker synergistic effect than with the 0.1% plant dose (Fig. 5). The lowest plant dose (0.001%) had no effect at all, except for a slight increase in take-flight. All the individual plant blend components (except for benzaldehyde) increased male response to a level not significantly different from the optimal 10% *Z*8-12:OH blend, but not significantly different from the suboptimal 0% *Z*8-12:OH blend (Fig. 5). Therefore the individual compounds (except benzaldehyde) synergized male responses, but their effect was not as strong as when presented as a blend.

4. Discussion

4.1. Effect of a plant volatiles on the response to overdosed pheromone blends

Our results agree with a previous study which explored both, the effect of pheromone concentration and ratio of *E*8-12:Ac on male response (Baker et al., 1981). Similar to that study, male *G. molesta* responses peaked to optimal pheromone concentrations and ratios of the two acetate isomers. We have previously shown that plant volatiles synergize male response to below-optimal pheromone doses (Varela et al., 2011), however in the present study we failed to observe plant synergism to above-optimal plant doses. This could be explained by the different mechanisms by which low and high pheromone doses reduced response levels. With low doses the olfactory system is understimulated and therefore the stimulus arriving to the CNS is probably below the behavioral response threshold. Plant odors, which in our test did not stimulate male flight on their own but that under natural conditions could signal the presence of conspecific females (Deisig et al., 2014), may lower the behavioral response threshold to pheromone (since the pheromone receptor neurons are unaffected by the presence of plat odors in the pheromone blend, Ammagarahalli and Gemeno, 2015), and so increase responses to below optimal pheromone doses. With high stimulus doses however, the olfactory system is sufficiently stimulated from the distance to arouse take flight and oriented flight, but males interrupt upwind progress (i.e., arrest) close to the odor source probably due to adaptation at the peripheral olfactory level (De Bruyne and Baker, 2008). Under these conditions the effect of the plant odor is probably negligible, given that the pheromone receptor neurons are probably adapted and unable to transmit a proper pheromone stimulus to the brain despite simultaneously processing an optimal plant signal. Schmidt-Büsser et al. (2009) report behavioral synergism to an overdose pheromone blend in the

tortricid *Eupoecilia ambiguella* Hübner, so in some cases the plant blend can cancel out the effect of a high pheromone dose, but more studies are needed to have a broader picture of this phenomenon.

4.2. Effect of plant volatiles on the response to suboptimal Z/E pheromone isomer ratios

The results of the present study support previous findings showing that the proportion of *E*8-12:Ac in the blend is critical for optimal male attraction (Linn and Roelofs, 1981; Willis and Baker, 1988; Knight et al., 2015). The plant blend reverted some of the negative effects of the unnatural low and high E-isomer ratios, mainly at the earlier stages of response (take off and oriented flight), but it failed to influence the response of the male when close to the pheromone source. This lack of effect of the plant blend on a suboptimal blend ratio of the main pheromone ingredients may reflect the strong selective pressure imposed by costly mating mistakes with species producing similar pheromone blends (De Bruyne and Baker, 2008; Bruce and Pickett, 2011). Our results suggest that although plant odors are able to compensate for unnatural pheromone blend ratios under laboratory conditions, the effect may be diluted under field conditions where plant volatiles are ubiquitous and therefore will mix with the pheromone stimulus (Deisig et al., 2014).

4.3. Role of alcohols and plant volatiles on sex pheromone response

Our study confirms that *Z*8-12:OH synergizes male response to a pheromone blend containing an optimal ratio of the two acetates but lacking alcohols, but whereas in a previous wind tunnel study synergism started already at the 3% *Z*8-12:OH dose (Linn and Roelofs, 1983) we did not observed it until the 10% dose. In addition, in our study the alcohols 12:OH and codlemone, as well as the plant blend, produced the same effect as *Z*8-12:OH, so this compound does not appear to be an essential ingredient in the pheromone blend of *G. molesta* since its role can be replaced by similar components. Cardé et al. (1975a,b) report that 12:OH acts only at the close-range, however we found that it significantly improved at all the stages of the behavioral male response in a wind tunnel. However a field test would be needed to confirm long-range responses to this compound under natural conditions. Baker and Cardé (1979) indicate that the role of the two alcohols (*Z*8-12:OH and 12:OH) depends on the presence of each other and on the ratio of *E*8-12:Ac to *Z*8-12:Ac in the blend, so further tests with more treatment combinations may show additional roles for these alcohols. *G. molesta* is not attracted to the sex pheromone of *C. pomonella*, E8,E,10-12:OH, but when this compound is mixed with its own pheromone it increases *G. molesta* captures, and their combined use is a new approach targeting both populations in the field (Evenden and McLaughin, 2005; Knight et al., 2014). In our test, unlike the previous ones, the effect of codlemone was tested in the absence of *Z*8-12:OH, and we show that codlemone effected similar levels of synergism as *Z*8-12OH, and interestingly both had the strongest effect at the 10% ratio.

The three alcohols have relatively similar chemical structures, so a generalistic alcohol ORN could be enough to detect the three of them. On the other hand, each alcohol molecule could have its own specific receptor. We have been unable to detect ORNs on the male antenna that are specifically tuned *Z*8-12:OH (Ammagarahalli and Gemeno,

2014), but whether there are receptors tuned to codlemone or 12:OH remains to be tested. It is unlikely, though, that male *G. molesta* would have a receptor specific for codlemone because the two species do not cross-attract as they do not share the main pheromone compounds (Knight et al., 2014). Plant volatiles do not excite pheromone ORN, instead they are perceived by general odor ORNs housed in other sensilla, mainly auricillica but also in some sensilla trichodea (Ammagarahalli and Gemeno, 2015). The synergism of the plant blend on the no-alcohol pheromone blend probably involves excitation of these plant ORNs. Varela et al. (2011), show that Z3-6:OH and benzonitrile, individually, synergize the response to a suboptimal pheromone blend. Because in our study Z3-6:OH was not more active than the other ingredients of the blend, it is unlikely that this compound alone was responsible for the synergistic effect of the complete plant blend. More likely the plant blend is perceived as an odor object by the integration of stimuli from different sensory neurons. Benzonitrile has been reported as having an important role in the response to plant odors (Najar-Rodriguez et al., 2010), but it did not synergize responses in our test.

Support for the importance of *Z*8-12:OH as an ingredient in the pheromone of *G. molesta* comes from studies showing that calling females release it (Baker et al., 1980), that males do not respond to a blend containing no *Z*8-12:OH, and that just a small percentage of the alcohol (1-3%) is needed to increase male attraction significantly (Baker and Cardé, 1979; Linn and Roelofs, 1983). By contrast, *Z*8-12:OH is not necessary for attraction (Roelofs and Cardé, 1974; Yang et al., 2002), its proportion in the blend can vary widely without affecting male response (Linn and Roelofs, 1983), and females do not release it (Lacey and Sanders, 1992). We compared pheromone composition and male response across worldwide populations of *G. molesta* and found little variation in the quantity of this compound in glands (Knight et al., 2014), but in comparison, other studies show little or no traces of *Z*8-12:OH in the female gland extractions (reviewed by Boo, 1998; El-Sayed and Trimble, 2002).

Closely-related species sharing similar pheromone blends, and therefore at risk of interspecific mating, may evolve olfactory signals designed to deter mutual attraction (Cardé and Haynes, 2004). *Z*8-12:OH inhibits males of two species that are closely related to and that use a similar ratio of the Z/E acetates as main pheromone ingredients as *G. molesta* [*Grapholita funebrana* (Treitschke) (Guerin et al., 1986), and *Grapholita prunivora* (Walsh) (Baker and Cardé, 1979)], so it is possible that the production and release of *Z*8-12:OH by *G. molesta* females may serve an interspecific avoidance function. In a similar fashion, two compounds in the pheromone glands of *G. funebrana* (Z8-14:Ac and Z10-14:Ac) do not play a role in attracting this species but they reduce captures of *G. molesta* (Guerin et al., 1986). Interestingly lesser captures of *C. pomonella* to mixtures of 2 pheromones (Knight et al., 2014)

4.4 Conclusions

Under natural conditions sex pheromones and plant odors mix in the air and together stimulate responding insects, however relatively little is known about the effect of plant odors on pheromone response, and vice versa. Plant volatiles reportedly synergize male moth responses to sex pheromone in several species, both under field and laboratory

conditions (Deisig et al., 2014), however few studies had explored the effect of plant odors on unnatural pheromone blends consisting of unbalanced component ratios or unusually high concentrations (Büsser-Schmidt et al., 2009). Our study shows that under laboratory conditions plant odors can offset some of the abnormal pheromone compositions tested, and that they can even play the role of missing minor pheromone ingredients, providing some resilience to the system. These findings pose new questions regarding perception and integration of pheromone and plant signals, and future studies should explore how the olfactory system perceives and integrates plant and pheromone information in order to understand the interplay between these two types of stimuli.

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Table 1. Chemical compounds used in the experiments

Compound (Abbreviation)	Ratio of each compound	CAS	Provider	Product number	Lot number	Purity (≥ %)
(Z)-3-hexenyl acetate (Z3HA)	70	3681-71-8	S. Aldrich	W317101	MKBD9967V	98
(Z)-3-Hexenol (Z3OH)	14	928-96-1	S. Aldrich	W256307		98
			Fluka	5306	1323459	98
(E)-2-hexenal (E2AL)	2	6728-26-3	S. Aldrich	W256005	19996MH	95
Benzaldehyde (BZA)	13	100-52-7	S. Aldrich	B1334		99
				12010	1412950	99
Benzonitrile (BZN)	1	100-47-0	S. Aldrich	12722	1293869	98

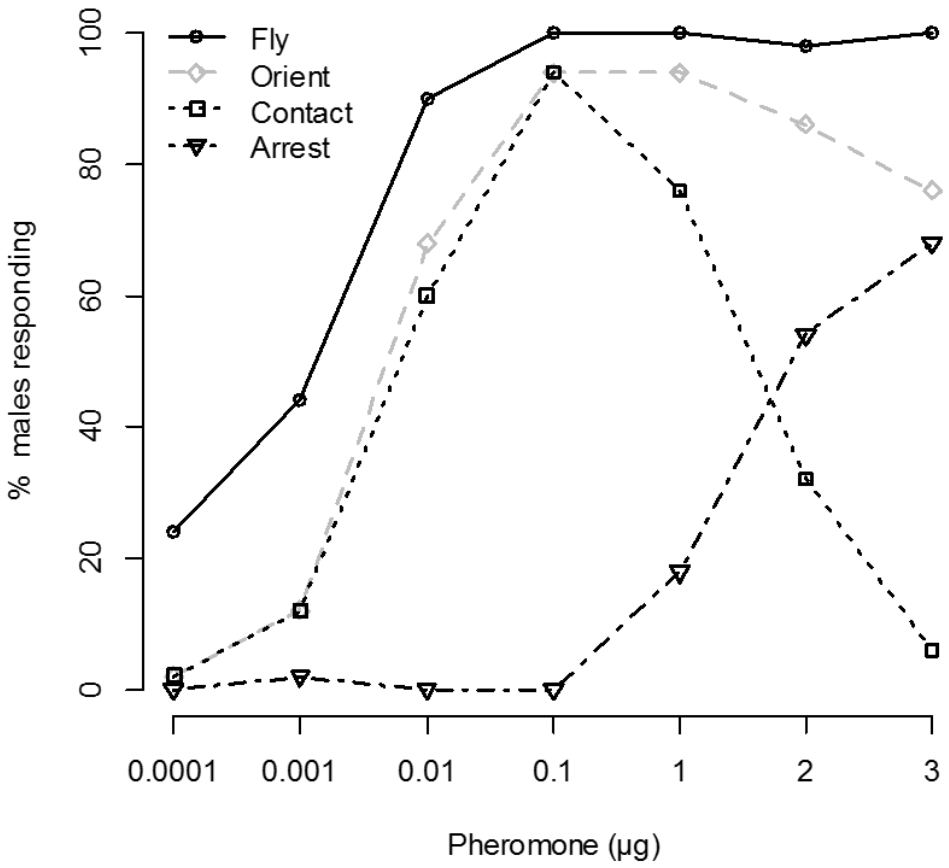

Figure 1. Effect of pheromone quantity on the wind tunnel response of *G. molesta* males.

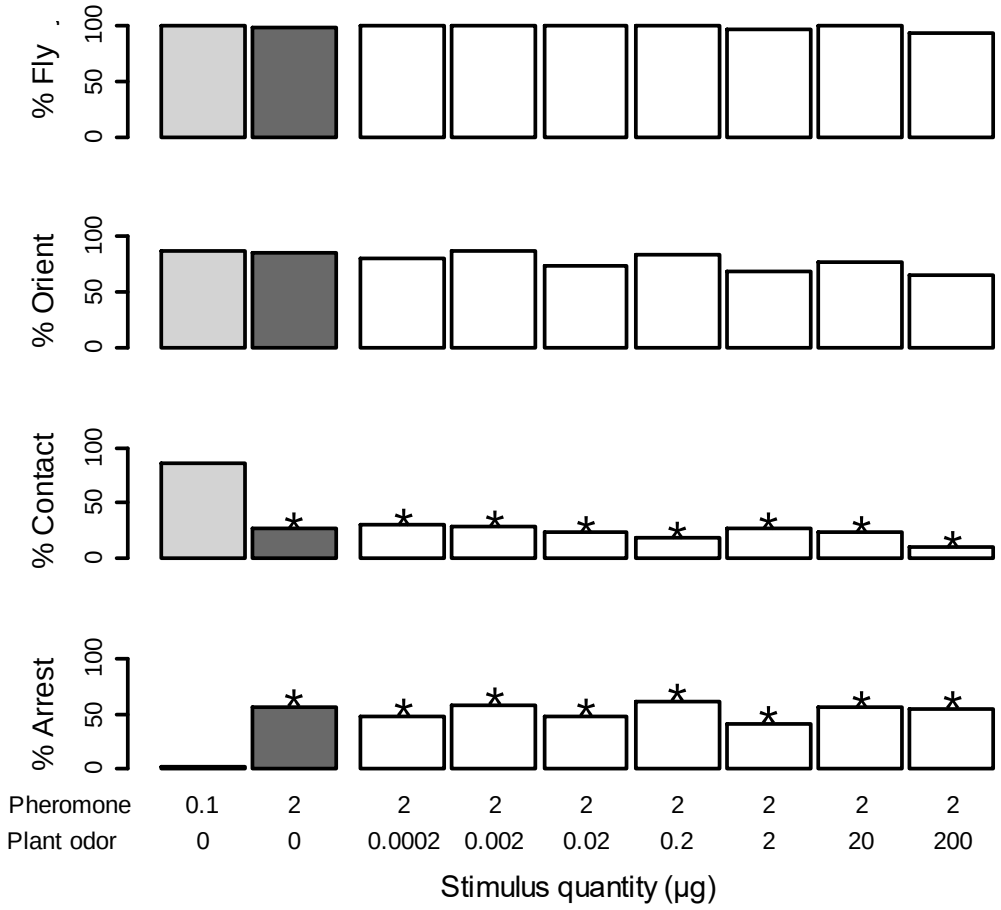

Figure 2. Effect of plant odor on the wind tunnel response of *G. molesta* males to overdosed sex pheromone. Males were exposed to and optimal pheromone dose (light grey box, 0.1µg), to an unnaturally high dose (dark grey, 2µg), and to the overdosed pheromone mixed with varying amounts of a plant odor (white columns). Percentages of males responding (take flight, oriented flight, contact, and arrested flight) are the predicted responses from the estimated parameters of general linear models (GLM). Asterisks indicate significant differences between the optimal sex pheromone and all other treatments by means of planned pair-wise comparisons using Tukey's test ($P < 0.05$).

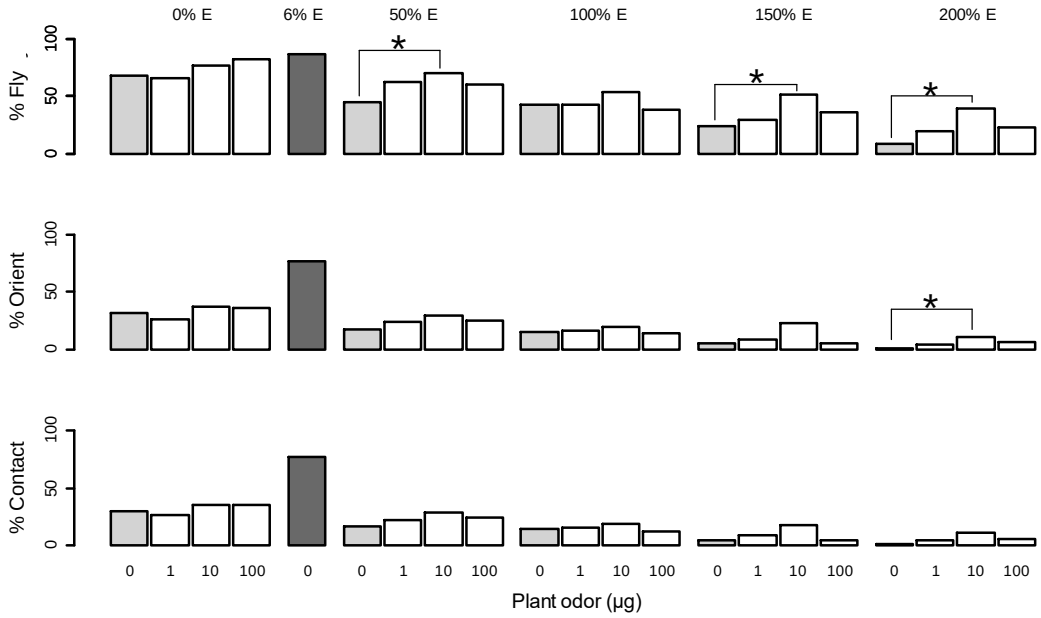

Figure 3. Effect of plant odor on the wind tunnel response of *G. molesta* males to sex pheromone blends containing a constant 100:10 ratio of *Z*8-12:Ac and *Z*8-12:OH (100:10 ng, respectively), and optimal ratio of the minor component (10% relative to the major component, dark grey column) or suboptimal ratios of *E*8-12:Ac (0, 6, 50, 100, 150 and 200%, light grey columns). The blends with suboptimal *E*8-12:Ac ratios were mixed with varying amounts of the plant odor (1:1, 1:10 and 1:100, major compound:plant odor respectively, white columns). Percentages of males responding (take flight, oriented flight, and contact) are the predicted responses from the estimated parameters of general linear models (GLM). Asterisks indicate significant differences between each unbalanced sex pheromone blend (light grey column) and the same blend with each of the plant odor concentrations treatments by means of pair-wise comparisons using Tukey's test ($P < 0.05$). The response to the optimal blend (dark grey column) was significantly higher than to any of the unbalanced E-blends (light grey columns, $P < 0.05$).

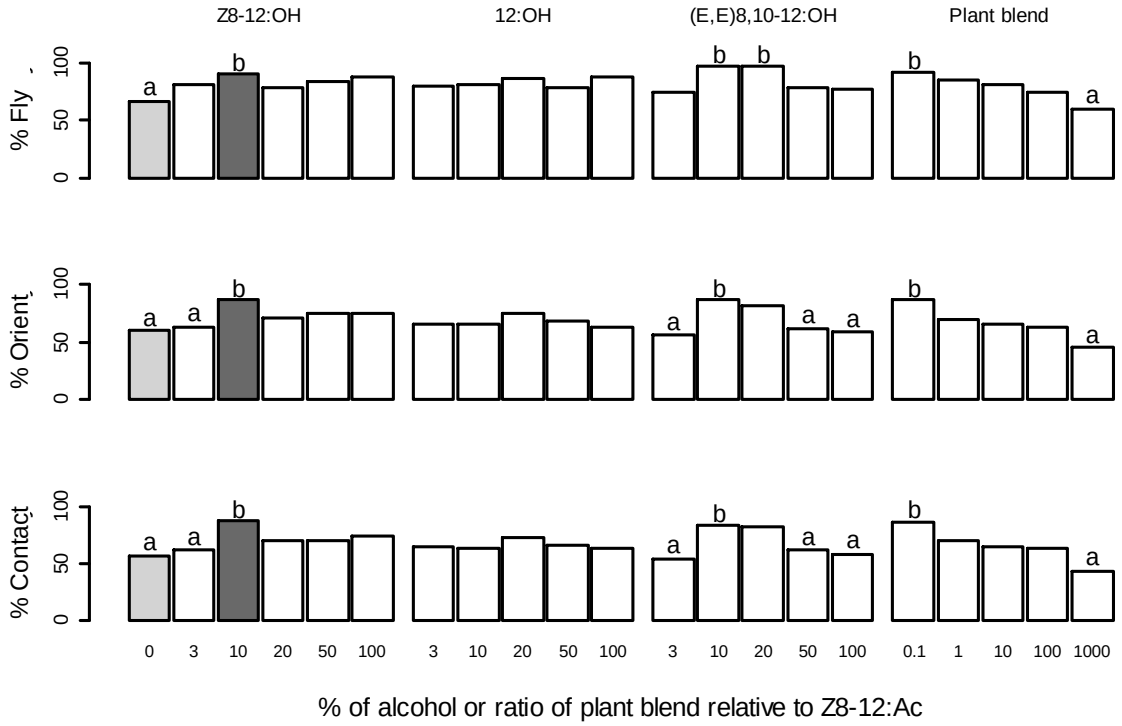

Figure 4. Effect of alcohols (*Z*8-12:OH, 12:OH, *E*8,*E*10-12:OH]) and plant odor on the wind tunnel response of *G. molesta* males to blends containing a constant 100:6 ratio of Z8-12:ac and E8-12:Ac (100:6 ng, respectively, light grey bar) mixed with several levels) of the alcohols (3, 10, 20, 50 and 100%, relative to *Z*8-12:Ac or the plant blend (1:0.1 to 1:1000 ratio, pheromone:plant blend, respectively). Percentages of males responding (take flight, oriented flight, and contact) are the predicted responses from the estimated parameters of general linear models (GLM). Planned pair-wise comparisons used Tukey's test ($P < 0.05$). "a" indicates significant differences between the optimal blend (10% *Z*8-12:OH, dark-grey bar) and each other treatment. "b" indicates significant differences between the suboptimal blend (no alcohol, light grey bar) and each other treatment.

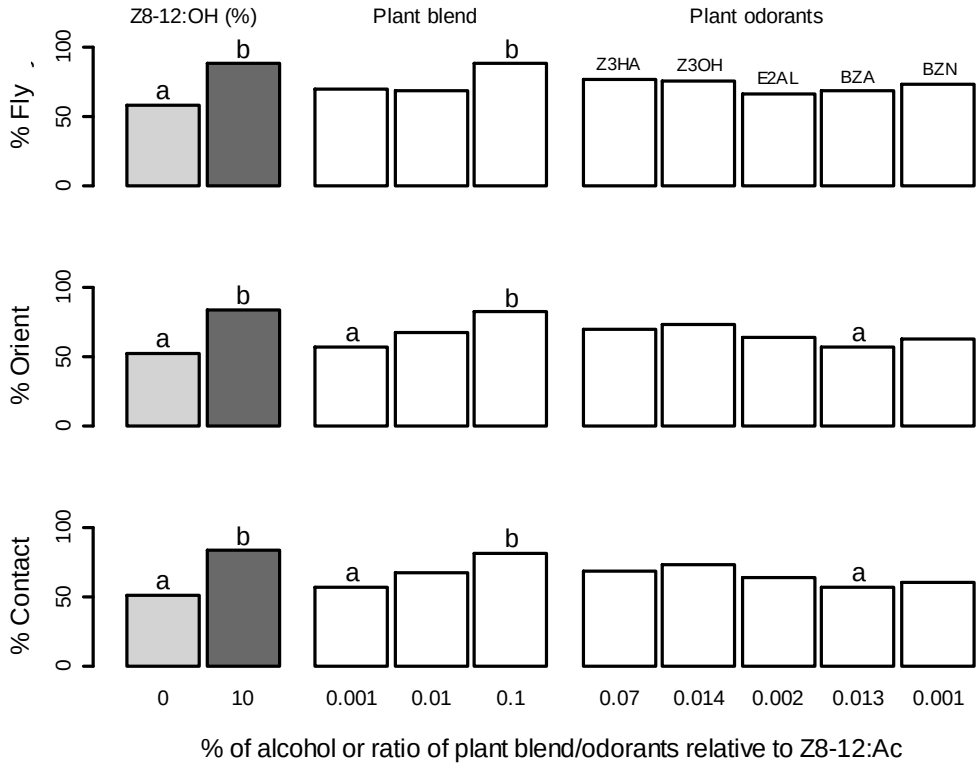

Figure 5. Effect of a plant odor blend and its individual components on the response of *G. molesta* males to blends containing a constant 100:6 ratio of Z8-12:ac and E8-12:Ac (100:6 ng, respectively) and no Z8-12OH (suboptimal blend, light grey bar), the acetate blend but with a 10% Z8-12:OH (relative to Z8-12:Ac, optimal blend, dark grey bar), and the acetate blend mixed with several ratios of the plant blend (1:0.001, 1:0.01 and 1:0.1, pheromone:plant, respectively), or with the individual plant ingredients in the same quantity as in the 1:0.1 pheromone:plant odor blend. Percentages of males responding (take flight, oriented flight, and contact) are the predicted responses from the estimated parameters of general linear models (GLM). Planned pair-wise comparisons used Tukey's test ($P < 0.05$). "a" indicates significant differences between the optimal blend (10% Z8-12:OH, dark-grey bar) and each other treatment. "b" indicates significant differences between the suboptimal blend (no alcohol, light grey bar) and each other treatment.

CHAPTER III

Response profile of pheromone receptor neurons in male *Grapholita molesta* (Lepidoptera: Tortricidae)

Response profile of pheromone receptor neurons in male *Grapholita molesta* (Lepidoptera: Tortricidae)

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Abstract

The response profile of olfactory receptor neurons (ORNs) of male *Grapholita molesta* (Busck) to the three female sex pheromone components [(*Z*)-8-dodecenyl acetate (*Z*8-12:Ac), (*E*)-8-dodecenyl acetate (*E*8-12:Ac), and (*Z*)-8-dodecenyl alcohol (*Z*8-12:OH)] was tested with single sensillum electrophysiology. Sensilla trichodea housed normally one, but sometimes two or three ORNs with distinct action potential amplitudes. One third of the sensilla contacted contained ORNs that were unresponsive to any of the pheromone components tested. The remaining sensilla contained one ORN that responded either to the major pheromone component, *Z*8-12:Ac ("Z-cells", 63.7% of sensilla), or to its isomer E8-12:Ac ("E-cells", 7.4% of sensilla). 31% of *Z*- and *E*-sensilla had 1 or 2 additional cells, but these did not respond to pheromone. None of the 176 sensilla contacted hosted ORNs that responded to *Z*8-12:OH. The proportion of *Z*- and *E*-cells on the antennae (100:11.6, respectively) is similar to the proportion of these compounds in the blend (100:6, respectively). The response of Z-cells was very specific, whereas E-cells also responded to the Z isomer, albeit with lower sensitivity.

Keywords: *Grapholita molesta*, single sensillum recording, sex pheromone, olfactory receptor neuron, sensillum.

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1. Introduction

Grapholita molesta (Busck) larvae bore on new growth shoots of peach trees (*Prunus* spp.) reducing fruit yield (Rothschild and Vickers, 1991). The sex pheromone has been described as a 100:6:10 blend of (*Z*)-8 dodecenyl acetate (*Z*8-12:Ac), (*E*)-8 dodecenyl acetate (*E*8-12:Ac), and (*Z*)-8 dodecenyl alcohol (*Z*8-12:OH), respectively (Roelofs et al., 1969; Beroza et al., 1973; Cardé et al., 1975a; Cardé et al., 1979; Baker and Cardé, 1979; Baker et al., 1981; Linn and Roelofs, 1983), and is used for monitoring and mating disruption over 50,000 hectares of peach and apple around the world (Witzgall et al., 2010).

The behavioural response of *G. molesta* to pheromone and plant odours has been studied in detail (e.g., Linn and Roelofs, 1981; Willis and Baker 1988; Linn et al., 1988; Linn et al., 1991; Willis and Baker, 1994; Piñero and Dorn, 2007; 2009; Il´ichev et al., 2009; Varela et al., 2011a; Lu et al., 2012; 2013; Najar-Rodriguez et al., 2012; 2013; Trimble, 2012). Electroantennography has been used to explore questions mainly related to mating disruption (e.g., Stelinski et al., 2006; Molinari et al., 2010; Trimble and Marshall, 2010; Khuns et al., 2012; D'Errico et al., 2013; Faraone et al., 2013), and at the CNS level, the three-dimensional structure of the antennal lobe (AL), and the physiological response of AL neurons to pheromone and plant odours have been studied (Najar-Rodriguez et al., 2010; Varela et al., 2009; 2011b). In addition Nagy and George (1981) and George and Nagy (1984) described the neuroanatomy of sensilla and olfactory receptor neurons (ORNs) in males, and Baker et al. (1988) analysed the effect of temperature on the ability of olfactory receptor neurons to detect pheromone pulses. However, a detailed characterization of the physiological response of pheromone receptor neuron types in *G. molesta* is lacking.

Pheromone ORNs make a large percentage of the receptor neurons on the male moth antenna and are extremely sensitive to low doses of sex pheromone (Kaissling, 2004). Electrophysiological studies in moths show specific pheromone component detection by distinct ORNs, which may be housed singly in a sensillum trichodeum or together with other pheromone ORNs (reviewed by De Bruyne and Baker, 2008; and Baker et al., 2012). In general, there is a correlation between the proportion of ORNs that respond to the major and minor pheromone components and the relative abundance of these compounds in the female-produced sex pheromone blend (Baker et al., 2012).

The aim of our study was to characterize the physiological response of male *G. molesta* ORNs to the three components of the sex pheromone blend. We mapped the position of different sensillum types on the antennae by SEM, and recorded the response of ORNs housed in sensilla trichodea to several doses of the pheromone components. We expected that males of *G. molesta* would have ORNs specific for each of the three pheromone components, and that these would be present in a proportion similar to the proportion of the pheromone components in the pheromone blend. Similarly, we expected that these ORNs would be highly sensitive and specific to their respective ligands.

2. Materials and Methods

2.1. Insects

The *G. molesta* colony originated from a laboratory rearing established at Piacenza, Italy, from insects collected in peach orchards in that locality, and was maintained at the University of Lleida, Spain, since 2005. Larvae were reared on a semi-synthetic diet modified from Ivaldi-Sender (1974) under a L16:D8 photoperiod at 25 ± 1ºC. Pupae were separated by sex and were placed in 4-L polypropylene containers provided with a cotton ball soaked in 10% sugar dissolved in water. Adults were separated daily and used when 2-4 days old. Care was taken not to expose adults to synthetic odour sources before the studies.

2.2. Scanning electron microscopy (SEM)

Male antennae were excised from the head with fine forceps. Scales were removed individually by hand under the stereomicroscope using a sharpened tungsten electrode, watching not to damage the sensilla hidden underneath. Antennae were mounted on SEM stubs lined with conductive double-side adhesive black tape, with the orientation of the mounted antenna to show the areas of interest. Preparations were air dried at room temperature for 3-4 days and then coated, using a sputter coater (Balzers SCD 050, Leica Microsystems, Madrid, Spain), with 50-nm gold particles for 3 min from a distance of 50 mm, with a current of 45 mA and Argon as cooling gas. Samples were examined in a scanning electron microscope (DSM 940A, Zeiss, Germany) at 10 kV and a working distance of 12 mm. The scale-free area of 9 antennae and the scaled area of 4 antennae, each from a different individual, were examined. Sensilla counts were made every 5th flagellomere, starting on the proximal one. Total sensilla count per antennae was estimated by extrapolating these counts to the other flagellomeres. The scale-free area, which covers one third of the perimeter of each flagellomere, was fully visible, but the scaled area, which covers the remaining of the flagellomere surface, was always partially obstructed from vision. Using characteristic landmark structures that indicated the sagital axis on the scaled area we could extrapolate sensilla counts from the visible section of the scaled area to the section hidden from view. Abundance and pattern of distribution of all types of sensilla are reported. Length, and basal and tip width of all types of sensilla (N = 20 sensilla from four different antennae) were measured.

2.3. Odourant stimuli

The pheromone compounds of *G. molesta*, (*Z*)-8-dodecenyl acetate (*Z*8-12:Ac), (*E*)-8-dodecenyl acetate (*E*8-12:Ac), and (*Z*)-8-dodecenyl alcohol (*Z*8-12:OH) were provided by Pherobank (The Netherlands) with an initial purity ≥ 99%. Gas chromatographic analysis revealed that *Z*8-12:Ac contained 0.38 % *E*8-12:Ac, and that *E*8-12:Ac contained 0.24% *Z*8-12:Ac. Undiluted compounds were weighted and diluted in n-hexane to make 100 µg/µl stock dilutions. Serial 10-fold dilutions of the stock dilutions in n-hexane were prepared from the stock solution as needed.

2.4. Electrophysiological recordings

Males were immobilized with industrial grade CO_2 for 10 s, and were mounted on a handcrafted poly(methyl methacrylate) insect holder. The body was inserted through a hole drilled in the holder and the protruding head was restrained by fixing a piece of adhesive cloth tape between the head and the holder. The antennae were carefully laid on a slant surface lined with double sided sticky tape, and were oriented for easy access with the electrodes. To record from sensilla located on the scaled area, scales were removed by gently rolling the antennae on the sticky tape. Remaining scales were removed individually with the help of a tungsten electrode. Sub-millimetric smoking paper strips placed over the antennae and glued to the sticky surface prevented antennal torsion. A stereo microscope (objective 2x, oculars 25x, zoom range 0.8-12.5, Leica Microsystems, Madrid, Spain) was used to help in these operations and to visualize the recordings. These were obtained by means of electrolytically (20% KNO_2) sharpened tungsten microelectrodes (0.125-mm diameter, 99.98% purity, Advent Research Materials Ltd, England). The reference electrode was inserted in the head through the mouth parts. For electroantennogram recordings (EAG) the tip of the recording electrode was inserted in one of the most distal segments of one antenna. For single sensillum recordings (SSR) the recording electrode was situated near the base of a randomly chosen sensillum trichodeum and pushed gently inward with the help of a manual micromanipulator (NMN-25, Narishige, Japan) until action potentials (AP) were detected. Flagellomeres 10 to 35 were sampled. The signal from the recording electrode was pre-amplified (10x gain, Universal Single Ended Probe, Syntech, Germany), filtered, and digitized (IDAC-4, Syntech, Germany), and recorded and analyzed in a PC (AutoSpike v.3.9, Syntech, Germany). The setup was mounted on an anti-vibration table (63-511, TMC Ametek, USA) and was shielded by a Faraday case to reduce low frequency noise.

2.5. Odour stimulation

Dilutions were applied as 1µl aliquots (1µl micropipettes, Drummond Scientific Co., USA) on 1 x 20 mm n-hexane-pre-cleaned filter paper strips (# 1, Whatman International Ltd, England). After having dried (5 min) the filter papers were introduced in n-hexane-pre-cleaned 100 µl glass micropipettes (1.2 mm internal diameter, Blaubrand® Intramark, Germany) which were then stored in glass test tubes sealed with PTFE-coated screw caps until used. New stimuli cartridges were prepared each day, and a given cartridge was not used for more than 10 stimulations. Air flow was generated by two diaphragm aquarium pumps connected to a 3-way solenoid valve (CS-55, Syntech, Germany). A 0.5 l/min flow of charcoal-filtered and humidified air blew continuously over the insect preparation through a 5-mm internal diameter plastic tube placed 15-20 mm from the preparation (air velocity at exit = 0.4 m/sec). The tip of the odour cartridge bearing the filter paper was positioned 0.4 cm down from the recording point and perpendicular to the direction of the continuous air flow. A 0.2 l/m charcoal-filtered room air flow was puffed through the odour cartridge to the recording area for 200 ms (air velocity at exit = 2.9 m/sec). The flow of continuous humid air was decreased by 0.2 l/min during the puff. Time interval between puffs was at least 60 s, but longer if needed to let

the spike activity return to pre-stimulation levels. A maximum of 5 cells were recorded per insect, and at least 30 min between two cell recordings were allowed. The air around the preparation was constantly renewed with an exhaust to minimize contamination. Test tubes were rinsed with acetone and heated at 250°C overnight before reused.

2.6. Dose-response

Preliminary tests determined the range of concentrations to be used in the dose-response tests. For EAG we used 10, 100 and 1000 ng of each pheromone compound. For SSR the ORNs were first challenged with a high dose of each pheromone component (100 pg of *Z*8-12:Ac, 1 ng of *E*8-12:Ac, and 1 ng *Z*8-12:OH) to determine their physiological type (cells were typically more sensitive to one pheromone component and less sensitive to the others, see Results), and then dose-response curves were established in ORNs with stable contacts and good signal to noise ratio. The order of stimuli was first the negative control (n-hexane), followed by low to high doses of the test compounds. For each cell the full range of concentrations was tested for the most sensitive compound, whereas the range of concentrations tested for the other two compounds depended on the cell type. "*Z*-cells" were very sensitive and specific to *Z*8-12:Ac and were challenged with the full range of concentrations of *Z*8-12:Ac, but only with the two highest dosages of the other two compounds (except for a subset of 4 to 16 cells that were tested with the full range of E8-12:Ac). "*E*-cells" were most sensitive to E8-12:Ac, and moderately sensitive to *Z*8-12:Ac, so they were tested with the full concentration range of the two acetate isomers, but only with the two highest concentrations of the alcohol. Non-linear regression functions were fitted to the observed data (Byers, 2013). To correct for the 0.38% of *E*8-12:Ac present in *Z*8-12:Ac, the regression function of *E*-cells stimulated with *E*8-12:Ac was used to estimate how much of the response of the *E*-cells to stimulation with *Z*8-12:Ac was due to the *E*8-12:Ac contaminant, and the estimated response to the contaminant was subtracted from the observed response to *Z*8-12:Ac. Non-linear regression parameters were calculated using self-starting functions in R software (Crawley, 2009; R Core Team, 2012).

2.7. Cross-adaptation

A cross-adaptation test was performed to determine if the dual response of *E*-cells to *E*8-12:Ac and *Z*8-12:Ac was the result of a) one ORN responding to both compounds, or b) two ORNs of equal spike amplitude sharing the same sensillum but each responding to one of the two isomers. Two pheromone cartridges were angled at 45 degrees of each other with the outlets pointing to the SSR preparation. Each cartridge was connected to a different air flow and solenoid valve so that they could puff independently with the same airflow conditions described above. Once contact was established with an ORN it was first stimulated with *Z*8-12:Ac and *E*8-12:Ac to determine its type (*Z*- or *E*-cell, see Results). For cross-adaptation a single 200-ms puff from the first cartridge was followed by a 100-ms inter-stimulus interval and then by a 200-ms puff from the second cartridge. All possible combinations of the E- and *Z*-isomers (*E* and *E*, *Z* and *E*, *E* and *Z*, *Z* and *Z*) were tested in a given cell. The position (left or right pipette) of the stimulus was randomized among replicates. *Z*8-12:Ac and *E*8-12:Ac were puffed at 100 pg and 1ng,

respectively, in both ORN types, because these concentrations resulted in similar spike frequency responses according to the dose-response curves on E-cells.

2.8. Spike analysis

When more than one spike size was detected they were sorted by amplitude. For each puff the number of spikes during a 1-sec pre-stimulation period was subtracted from the number of spikes during a 1-sec post-stimulation period. Peri-stimulus time histograms (PSTH) were plotted by grouping spikes in 25-ms bins starting at the onset of stimulation. The response to *Z*8-12:Ac was more tonic in *Z*-cells than in *E*-cells. To determine if this difference was significant we calculated the times of half-rise and half-fall PSTH peak response, relative to the spontaneous activity of the cell (averaged for 1 sec pre-stimulation). PSTHs of *Z*-cells and *E*-cells to 100 pg and 1 ng loads of *Z*8-12:Ac, respectively, were normalized relative to the peak response, and 2nd order polynomial equations were fitted to the rise and fall phases. Estimated half-rise and half-fall times of cells stimulated with *Z*8-12:Ac were compared between *Z*- and *E*-cells with t-tests.

3. Results

3.1. Morphology

The antenna of *G. molesta* males is filiform and consists of 2 basal segments, scape and pedicel, and a flagellum composed of 45 flagellomeres, with no variation in number of flagellomeres among the 13 antennae from 13 different males analyzed. The flagellum carries most of the sensilla on the antenna. The dorsal and lateral areas of the flagellum bear scales, while a ventral band running the entire length of the flagellum (about 30% of the flagellomeres surface) remains scale-free. The apical flagellomere does not bear any scales.

Scanning electron microscopy of antennae revealed 6 different types of sensilla on the flagellomeres: trichodea, chaetica, coeloconia, auricillica, basiconica and styloconica (Figs. 1 and S1). The different types varied in distribution and density along the flagellum and between scaled and scale-free areas (Fig. S2). Sensilla trichodea were thin and long, (Table S1) and were surrounded by a socket-like structure. There were 2,291 sensilla trichodea per antennae (1,015.56 ± 78.71 and 1,276 ± 42.84, mean ± SEM, in scaled and scale-free areas, respectively), which constituted 72% of all the sensilla (Table S2). Their number increased steeply between flagellomeres 1 and 5, remained high between flagellomeres 5 and 35, and decreased steeply from flagellomere 35 towards the distal end of the antenna (Fig. S2). The average number of sensilla trichodea per flagellomere in flagellomeres 5 to 35 was similar in the scaled and scale-free areas (mean ± SEM, 34.9 ± 0.4 and 34.9 ± 0.6, respectively), and therefore sensilla trichodea were denser in the smaller scale-free area than in the twice larger scaled area (Table S2, Fig. 1). Spatial distribution of sensilla trichodea in a flagellomere was random in the scale-free area, and arranged in rows in between the scales in the scaled area sensilla auricillica (Table S2, Figs. 1 and S2). These are much shorter than sensilla trichodea and flattened rather than cylindrical, with a more variable range of sizes and shapes than sensilla trichodea (Table S1, Figs. 1 and S1). Sensilla auricillica constituted 11% of the total

sensilla in the antenna and were more abundant in the scaled area than in the scale-free area (Table S2, Figs. 1 and S2). They always occurred on the distal area of the flagellomere, being (Fig. 1). The second most abundant type of sensilla on the antennae of *G. molesta* males was more numerous on the lateral sides of the scaled area, and lacking in the central section of the scaled area (Fig. 1). The third most abundant type of sensilla (7% of the total sensilla) were the coeloconica (Table S2). These are easy to recognize by the central dome surrounded by 12-13 finger-like projections (microtrichia) (Figs. 1 and S1). Their distribution overlapped with sensilla auricillica. Sensilla basiconica made only 4 % of the total sensilla. They look similar to sensilla trichodea but are comparatively shorter and wider (Table S2, Fig. S1). They are present both in scaled and scale-free areas of the antennae.

The remaining two types of sensilla, chaetica and styloconica, were present in constant number and position in each flagellomere and served as topographic landmarks (Fig. 1). Each flagellomere, except the apical, bears one sensillum styloconica at the distal end of the mid-ventral area (Fig. 1). It consists of a finger-like structure, with a large pore at the terminal end (Fig. S1). Sensilla chaetica are similar to sensilla trichodea but they can be distinguished from the former ones because sensilla chaetica have a bulbous socket at the point of insertion on the antenna and they are more electron-dense and perpendicular to the surface of the antennae than the trichodea. There are four sensilla chaetica in the equator of each flagellomere, two on the laterals of the scaled area, and two on the scale free area (Fig 1).

3.2. Specificity and sensitivity of pheromone ORNs

3.2.1. ORN types

Sensilla trichodea can be categorized in three distinct groups based on the response of their ORNs to pheromone stimuli. One group of sensilla housed a cell that was very sensitive to the major pheromone component, *Z*8-12:Ac, and responded very little to the highest concentrations of *E*8-12:Ac and *Z*8-12:OH (Fig. 2A). These were called *Z*-sensilla and *Z*-cells. A second type of sensilla contained a cell that was most sensitive to *E*8-12:Ac, showed intermediate response to *Z*8-12:Ac, and responded very little to the highest concentrations of *Z*8-12:OH (Fig. 2B). These were called *E*-sensilla and *E*-cells. *Z*- and *E*-sensilla made 63.6 % and 7.4% of the sensilla trichodea on the antennae, respectively (Table S3). The rest of the sensilla (29%) contained cells that did not respond to any of the three pheromone components, as determined with a single high-concentration pheromone puff (Table S3). Out of 176 sensilla sampled for their response to the three pheromone components, we did not find a single ORN that responded to *Z*8-12:OH with similar sensitivity as the *Z*- and *E*-cells to their own ligands.

Z-sensilla were located along most of the length of the flagellomere (flagellomeres 10 to 35), whereas E-sensilla were usually located in the distal mid-dorsal (scaled) and proximal mid-ventral (scale-free) areas of the flagellomere. The proportion among sensilla types (Z, E and non-responding) was independent of flagellum area (scaled or scale-free) ($\chi 2 = 4.04$, df = 2, $P > 0.132$, Table S3). The proportion of *Z*-sensilla and sensilla with non-responding cells was significantly higher in the scale-free area than in

the scaled area ($\chi2 = 6.68$, df = 1, P = 0.009, and $\chi2 = 7.44$, df = 1, P = 0.006, respectively), whereas E-sensilla were equally represented in both regions ($\chi2 = 1.99$, df = 1, P = 0.16) (Table S3).

3.2.2. ORN number, spontaneous activity and spike amplitude

More than half (62.5%) of all the sensilla trichodea housed a single neuron of large spike amplitude (mean ± SEM, 1.81 ± 0.17 mV), whereas a smaller percentage (21.02%) housed two neurons, where one was of large amplitude, similar to that of the single neurons, and the second one of a smaller spike amplitude, and the remaining 16.48% sensilla housed 3 neurons (Tables S3 and S4). 84% of the Z-sensilla had a single neuron, whereas the percentage was lower in E-sensilla (54%) and still lower in sensilla with unresponsive cells (33%) (Table S3). The proportion of Z-sensilla with more than one ORN was significantly higher in the scale-free area than in the scaled area ($\chi2 = 8.64$, df = 1, P = 0.003) (Table S3). Only one of the ORNs responded to pheromone in pheromone sensilla with more than one cell, and in 84% of the cases it was the large amplitude neuron (Tables S3 and S4). The spike amplitude and spontaneous activity of Z- and E-cells was similar to each other and between single and paired cells (Table S4). Unresponsive cells were more variable in amplitude and spontaneous activity than the pheromone cells. 60% of the unresponsive cells had large spike amplitudes, as in pheromone cells (> 1 mV), whereas the remaining 40% had small spike amplitudes (< 1 mV). The spontaneous activity of 73% of the unresponsive cells was high (> 10 AP/sec), whereas in the remaining 27% the spontaneous activity was comparable with that of pheromone cells (< 10 AP/sec) (Table S4).

3.2.3. Dose-response

The response of Z-cells to *Z*8-12:Ac and of E-cells to *E*8-12:Ac was sigmoidal in shape in the log-concentration scale, with a response intensity similar to n-hexane control up to the 1 pg stimulus concentration, rising steeply up to 1 ng stimulus concentration and starting to balance off at the 10 ng stimulus concentration (Fig. 2). n-hexane produced minute changes in spontaneous activity (Fig. 3). When the effect of the 0.38% contamination of E8-12:Ac in *Z*8-12:Ac was corrected, the response of E-cells to *Z*8-12:Ac decreased by about half, but it still was comparatively larger than the response of these cells to *Z*8-12:OH, or than the response of Z-cells to *E*8-12:Ac (Fig. 2B).

Electroantennogram recordings showed significantly higher responses to the 3 pheromone components than to n-hexane at the highest stimulus concentration tested (1 µg; planned contrasts between each compound and n-hexane following a GLM for each concentration, P < 0.05; Fig. S3). At the two lower stimulus concentrations tested, the two acetates stimulated the antennae more than hexane, but the response to the alcohol was similar to hexane.

3.2.4. Cross-adaptation

The dual response of E-cells to *E*8-12:Ac and *Z*8-12:Ac could result from a single neuron responding to the two compounds, or from two neurons (of identical spike amplitude) but each responding to a different isomer. A cross-adaptation test in E-cells,

showed no response to the second puff in any of the cross-compound combinations tested, which is in agreement with the hypothesis that E-cells consist of just one cell responding to both isomers (Fig. 3A). The cross-adaptation response was indistinguishable from the response to two consecutive puffs of the same isomer. In contrast, in Z-cells a puff of *Z*8-12:Ac following a puff of *E*8-12:Ac produced a response during the second puff, but not during the first puff, as is expected from a single cell responding just to *Z*8-12:Ac (Fig. 3B).

3.2.5. Response duration of Z- and E-cells to Z8-12:Ac

Although both Z- and E-cells responded to *Z*8-12:Ac, the temporal pattern of response was different between them (Fig. 4). After stimulation, spontaneous activity rose sharply to peak frequency, and then decreased to spontaneous activity level in both cell types. The time after puff onset and half-rise was similar in Z- and E-cells (mean ± SEM, 0.148 ± 0.005 s and 0.147 ± 0.002 s, respectively; t-test, $P = 0.410$), but Z-cells remained active for a longer time as indicated by a longer time to half-fall in these cells (mean ± SEM, 0.299 ± 0.004 s) than in the E-cells (mean ± SEM, 0.224 ± 0.003 s; t-test, $P < 0.001$). *E*8-12:Ac ORNs displayed similar dynamics to the two pheromone compounds.

4. Discussion

4.1. Sensilla morphology

The six morphological sensilla types of *G. molesta* are similar to those reported in other tortricids (Wall, 1978; Razowski and Wojtusiak, 2004; Ansebo et al., 2005), and Lepidoptera in general (Hansson, 1998). The total number of sensilla trichodea that we recorded, however, is much lower than what George and Nagy (1984) report (4,382 and 9,095, respectively, for the sum of the two antennae). Nagy and George (1981) show that total counts of sensilla trichodea in *G. molesta* vary up to 30% among individuals reared under different conditions, so the different number of sensilla between studies could be related to this factor. The percentage of sensilla trichodea relative to other sensilla types that we observed is similar to the 81% reported by George and Nagy (1984).

Sensilla auricillica and coeloconica were the next most abundant sensilla types on the antennae of *G. molesta* males. They are probably involved in the detection of plant odours and other organic compounds (Ebbinghaus et al. 1997; Ansebo et al., 2005; Wall, 1978). George and Nagy (1984) distinguish two types of sensilla basiconica on the antennae of *G. molesta* males, however we could identify only one type. They mapped the longer type I basiconica to the distal half of the scale-free area, and the shorter type II to the proximal half of the scaled area. In our observations sensilla basiconica were all located in the distal half of the flagellomere, and corresponded in length and number with sensilla basiconica type I of George and Nagy (1984), so they are probably the same type of sensillum. We, however, could not find sensilla basiconica type II of George and Nagy (1984), perhaps because it is shorter than sensilla basiconica type I. The arrangement of four sensilla chaetica in the equator of the flagellomere and one sensillum styloconicum at the tip is common in other tortricids (Wall, 1978; Ebbinghaus et al., 1997; Maher and Thiery, 2004; Ansebo et al., 2005).

4.2. Distribution of pheromone ORN types

Unlike what we expected, the ORNs tuned to the major pheromone component *Z*8-12:Ac are housed in different sensilla trichodea than the ORNs tuned to the stereoisomer, *E*8-12:Ac. Similar sensilla partition of ORNs is found in some noctuid moths, but in the other tortricids investigated, and in many other moth species, major component ORNs share sensilla with minor component ORNs (reviewed in De Bruyne and Baker, 2008; Baker et al., 2012). It has been proposed that the adaptive function of co-localized ORNs is related to the physiological constraint imposed by real time detection of precise odourant blend ratios (Baker et al., 1998; Baker et al., 2012; Binyameen et al., 2014). The question remains as to why in some species like *G. molesta*, pheromone ORNs are not co-localized, whereas in others like Ostrinia nubilalis (Hübner) they share sensillum with other pheromone ORNs (Domingue et al., 2007).

As a general rule, when ORNs are co-localized the major component ORN has a larger dendrite size, whereas sensilla with a single ORN responding to the major component are more abundant than sensilla housing neurons tuned to minor components (Baker et al., 2012). The second case applies to *G. molesta* because major and minor component ORNs occur in different sensilla, and the major component ORNs are more abundant than minor component ORNs, whereas the spike amplitude of both ORN types is relatively similar, which is an indication of similar dendrite size between them (Hansson et al., 1994).

4.3. Pheromone-unresponsive ORNs

A relatively large percentage (29%) of the sensilla trichodea in *G. molesta* males house ORNs that do not respond to the pheromone components. In male *Manduca sexta* (L.) 59% of the sensilla trichodea host ORNs responsive to sex pheromone components, whereas the rest either respond to plant odours (20%) or do not respond to any test compound (21%) (Kalinová et al., 2001). In male *Agrotis segetum* (Schiff.) (Hansson et al., 1989) and Heliothis subflexa (Guenée) (Baker et al., 2004) relatively smaller percentages of unresponsive ORNs were reported in sensilla trichodea (11% and 2%, respectively). Male *G. molesta* respond behaviourally to plant volatiles (Varela et al., 2011a; Il'ichev et al., 2009; Lu et al., 2013), so some of their unresponsive ORNs could be tuned to plant volatiles. In addition, ORNs unresponsive to pheromone compounds could be used to detect pheromone compounds from other species that inhibit male *G. molesta* response to the female pheromone, such as Z6-12:Ac and Z10-14:OH (Guerin et al., 1986; Tòth et al., 1991), as happens in other species (reviewed in De Bruyne and Baker, 2008).

4.4. Ligand specificity of pheromone ORNs

Male *G. molesta* behaviourally discriminate small variations in the ratio of the two acetate isomers (Baker and Cardé, 1979; Baker et al., 1981; Knight et al., 2014a), so they should have a detection system that reports the relative abundance of the two isomers in the blend. In the majority of moths this is achieved by having specific receptor neurons to each of the main pheromone components (De Bruyne and Baker, 2008). However in *G. molesta* the ORNs tuned to the minor component (*E*8-12:Ac) also respond to the major

component (*Z*8-12:Ac). The 0.38% *E*8-12:Ac contamination in the *Z*8-12:Ac solution contributed only slightly to the unspecific response of the E-cells, as we demonstrated after subtracting its effect. The cross-adaptation test indicated that sensilla housing the E-cell did not house a second ORN responding to *Z*8-12:Ac, and confirms that a single and not very specific ORN responds strongly to *E*8-12:Ac and less to *Z*8-12:Ac.

Low specificity ORNs for key pheromone components have been scarcely reported in the literature (Takanashi et al., 2006; Domingue et al., 2008). In *O. furnacalis* (Guenée) a large proportion of the ORNs respond equally well to the two main components (*E*12-14:OAc and *Z*12-14:OAc) (Takanashi et al., 2006). However, unlike *G. molesta*, O. furnacalis also has ORNs specifically tuned to each pheromone component. So, how to explain the apparent absence of *E*8-12:Ac-specific ORNs in *G. molesta*? One possible explanation is that *E*8-12:Ac-specific ORNs are rare and were missed in our sample of 176 sensilla. Very low frequencies (< 2%) of ORNs tuned to pheromone components have been reported in other species (Hansson et al., 1990; Quero et al., 2004).

Another possibility is that fully specific ORNs are not essential for pheromone blend discrimination in *G. molesta*. To explain this point we must assume that each ORN type (Z and E) innervates a different glomerulus, as happens in most moth species (Lei and Vickers, 2008), and that both glomeruli will be excited by *Z*8-12:Ac, but more intensely the *Z*8-12:Ac than the *E*8-12:Ac glomerulus, due to the larger number of *Z*8-12:Ac ORN axons innervating it. Because *E*8-12:Ac will excite only the *E*8-12:Ac glomerulus, departures in the relative response of the two glomeruli to a blend of both isomers, with respect to excitation with *Z*8-12:Ac alone, will inform the insect of the presence of *E*8-12:Ac in the blend. Differential glomerular excitation could, thus, report stimulus composition even when one of the two ORNs is not fully specific. To confirm this point we should determine the presence of specific glomeruli for the Z- and E-ORNs. Male *G. molesta* have one large glomerulus at the entrance of the AL that is lacking in females (Valera et al., 2009), so it is very likely that this glomerulus is innervated by *Z*8-12:Ac-specific ORNs. Antennal retrograde staining coupled with electrophysiological recordings (Hansson et al., 1992) could confirm if each ORN type innervates a different glomerulus, and calcium imaging (Piñero et al., 2007) could measure the relative response ratio of the glomeruli to different pheromone blends.

The different temporal response dynamics of Z- and E-cells in response to *Z*8-12:Ac that we observed (longer lasting response in Z-cells), could provide odour identity information to the brain, but how this could help the brain to discriminate between Z-and E-excitation in E-cells is not clear. Although it is generally accepted that pheromone receptor neurons are highly specific (reviewed by De Bruyne and Baker, 2008), relatively few studies have determined the specificity of the most behaviourally relevant pheromone component ORNs for a given species. Additional studies of ORN specificity are needed to determine if species bearing low-specificity pheromone ORNs, like *O. furnicalis* and *G. molesta*, are more common than generally assumed.

4.5. Detection of Z8-12:OH

One unexpected result from our study was the apparent absence of *Z*8-12:OH-specific ORNs because this compound is emitted by females and affects male attraction (Cardé et al., 1975a; Cardé et al., 1979; Baker and Cardé, 1979; Baker et al., 1980; Linn and Roelofs, 1983), and in our EAG tests it stimulated the antenna. *Z*8-12:OH is 10% of the pheromone blend, so it probably has few dedicated ORNs that were missed in our sampling of 176 sensilla, or these neurons were not very specific or housed in sensilla other than trichodea (e.g. sensilla auricillica in *Cydia pomonella* (L.), Ebbinghaus et al., 1997; Ansebo et al., 2005), which were not sampled in our study. Whereas narrow variations in the ratio of the two acetates have a strong effect in male behavioural response (Baker et al., 1981; Knight et al., 2014a), males accept wide variations in the quantity of *Z*8-12:OH in the blend (Baker and Cardé, 1979; Linn and Roelofs, 1983, Linn et al., 1986), and in some locations *Z*8-12:OH is produced in trace amounts by females (Lacey and Sanders, 1992), or does not seem to play a role in attraction (Han et al., 2001; Jung et al., 2013). Furthermore, other alcohols affect the response of male *G. molesta* to the two acetates (Baker and Cardé, 1979; Cardé et al., 1975a; 1975b; 1979), including the sex pheromone of *C. pomonella* (Knight et al., 2014b), and *Z*8-12:OH inhibits males of closely related species (*G. funebrana* (Treitschke) and *G. prunivora* (Walsh)), that use a similar ratio of the Z/E acetates as *G. molesta* (Baker and Cardé, 1979; Guerin et al., 1986), so a reinvestigation of the role of alcohols in the olfactory communication of *G. molesta* is warranted.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jinsphys.2014.10.011.

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Table S1. Morphometry (μm ± SEM) of sensilla types on male *G. molesta* antennal flagellum (N=20). [1] In coeloconica only measured the central part.

Sensillum type	Length	Basal width	Tip width
Trichodea	38.86 ± 0.28	1.63 ± 0.02	0.42 ± 0.00
Auricillica	13.73 ± 0.46	1.97 ± 0.02	1.86 ± 0.03
Coeloconica[1]	6.09 ± 0.07	1.16 ± 0.05	0.27 ± 0.01
Basiconica	12.86 ± 0.37	1.70 ± 0.02	0.77 ± 0.05
Chaetica	31.68 ± 0.02	1.59 ± 0.02	0.49 ± 0.00
Styloconica	19.25 ± 0.23	3.68 ± 0.07	2.51 ± 0.04

Table S2. Number (mean ± SEM) of morphological sensillum types on scaled and scale-free areas of the antennal flagellum of *G. molesta* males. Sum of scaled and scale-free areas is not averaged because counts from scaled and scale-free areas come from different sets of antennae.

Sensillum type	Scaled area (n=9)		Scale-free area (n=4)		Total	
	number	%	number	%	number	%
Trichodea	1,015.56 ± 78.71	65.89 ± 1.20	1,276 ± 42.84	78.63 ± 0.09	2,291.5	72.11
Auricillica	294.67 ± 22.59	19.13 ± 0.61	53.75 ± 11.25	3.28 ± 0.62	348.42	10.96
Coeloconica	127.77 ± 16.98	8.02 ± 0.63	95 ± 4.56	5.86 ± 0.31	222.78	7.01
Basiconica	36.67 ± 6.48	2.38 ± 0.44	76.25 ± 4.73	4.71 ± 0.33	112.92	3.55
Chaetica	80.55 ± 2.94	5.36 ± 0.25	81.75± 4.66	5.02 ± 0.12	162.31	5.18
Styloconica	0	0	40 ± 2.04	2.46 ± 0.09	40	1.26
Total	1,534.38 ± 114.09		1,622.75 ± 55.66		3,168.08	

Table S3. Number of sensilla trichodea types in the antennal flagellum of *G. molesta* males according to the number of ORNs present per sensillum (1, 2 or 3), the response of the ORNs (Z, E or unresponsive), and their location (scaled or scale-free areas) (N = 20 and 39 individuals, respectively). Z and E-ORNs were housed in separate sensilla. Notice the high proportion of pheromone unresponsive sensilla (28.9%), and the larger number of Z-cells housed in 2-ORN sensilla in the scale-free area than in the scaled area. The ratio of Z:E cells (100:11.6) corresponds approximately to the ratio of these pheromone components in the pheromone blend (100:6).

Sensillum type	ORN/ sensillum	Scaled area	Scale-free area	Total (%)
Z-type	1	48	38	86
	2	0	19	19
	3	0	7	7
	Total (%)	48/63 (76.1%)	64/113 (56.6%)	112/176 (63.6%)
E-type	1	4	3	7
	2	3	3	6
	3	0	0	0
	Total (%)	7/63 (11.1%)	6/113 (04.4%)	13/176 (07.4%)
Unresponsive	1	5	12	17
	2	3	9	12
	3	0	22	22
	Total (%)	8/63 (12.6%)	43/113 (38.0%)	51/176 (28.9%)

Table S4. Spike amplitude and spontaneous activity of ORNs in male *G. molesta* antennae according to their response type (Z, E and unresponsive) and to the number of ORNs per sensillum. The ORNs in Table S4 include some ORNs from Table S3.

Cell type	ORN/ sensillum	Amplitude (mV)	Spontaneous activity (AP/sec)	Respond to pheromone
Z-type	1	1.81 ± 0.17	7.44 ± 0.84	25/25
	2	1.78 ± 0.22	4.41 ± 0.65	12/12
		0.83 ± 0.19	13.33 ± 3.05	0/12
	3	2.79 ± 0.32	2.57 ± 1.44	3/7
		1.72 ± 0.26	7.57 ± 1.08	4/7
		0.79 ± 0.16	16.28 ± 4.88	0/7
E-type	1	1.57 ± 0.09	7.82 ± 0.84	23/23
	2	1.73 ± 0.26	5.5 ± 1.05	6/6
		0.47 ± 0.06	25.66 ± 14.79	0/6
Unresponsive	1	1.15 ± 0.14	11.0 ± 2.9	0/16
	2	1.54 ± 0.19	5.83 ± 1.96	0/12
		0.79 ± 0.15	22.41 ± 6.87	0/12
	3	2.02 ± 0.24	5.41 ± 1.92	0/12
		1.17 ± 0.19	17.16 ± 4.06	0/12
		0.63 ± 0.14	14.66 ± 3.32	0/12

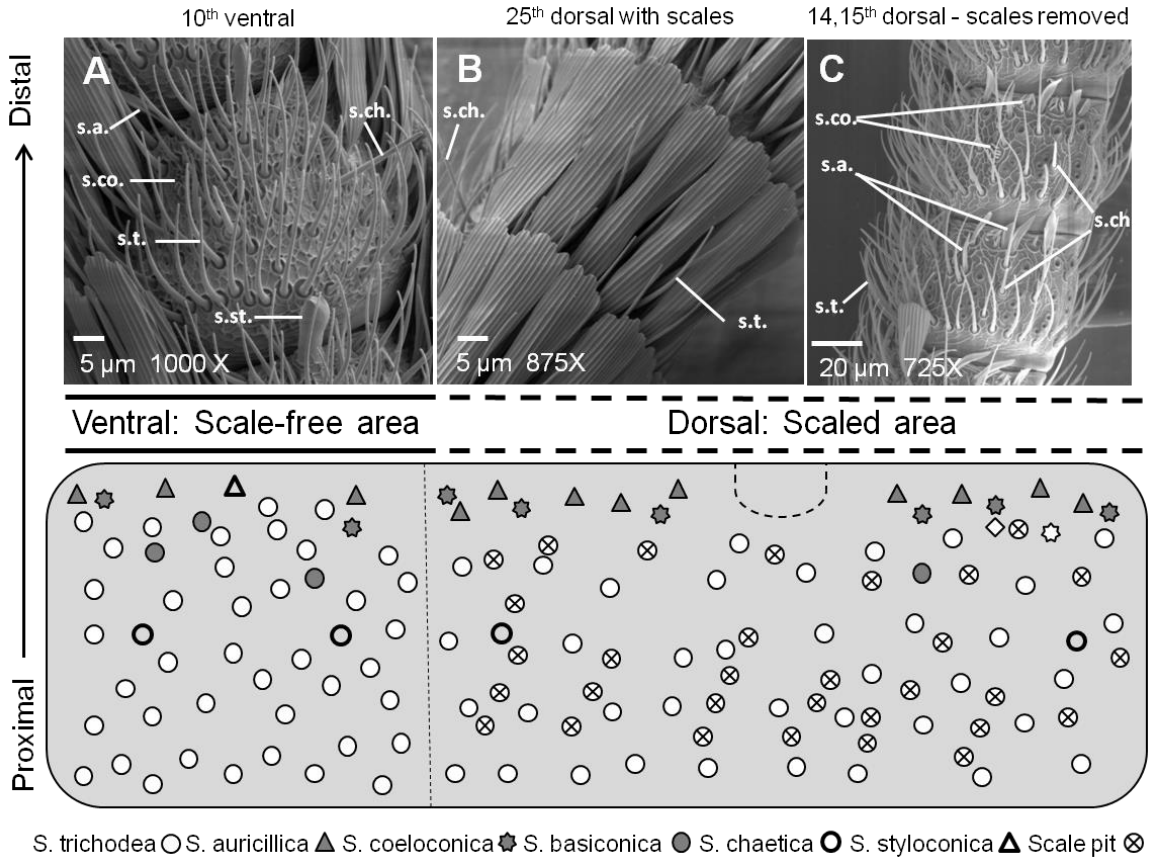

Figure 1. Distribution of sensilla types on the flagellum of *G. molesta* males. The top part of the graph shows SEM pictures of (A) the scale-free ventral region, (B) the dorsal region, which is covered with scales, and (C) the dorsal region when the scales have been removed. All sensillum types are seen on the scale-free ventral region (except the basiconica which are not visible in this SEM figure), whereas the scale-bearing dorsal region shows only sensilla trichodea and chaetica (B). All other sensillum types (except styloconica, and the basiconica in this SEM picture) are visible in the scaled area when the scales are removed (C). The bottom part of the graph shows a schematic representation of the number and position of the different sensilla types for a prototypical flagellomere. The dashed line in the scaled area indicates a protuberance on that area of the flagellomere. Sensilla auricillica (s.a), sensilla basiconica (s.b), sensilla chaetica (s.ch), sensilla coeloconica (s.co), sensilla styloconica (s.st.), and sensilla trichodea (s.t).

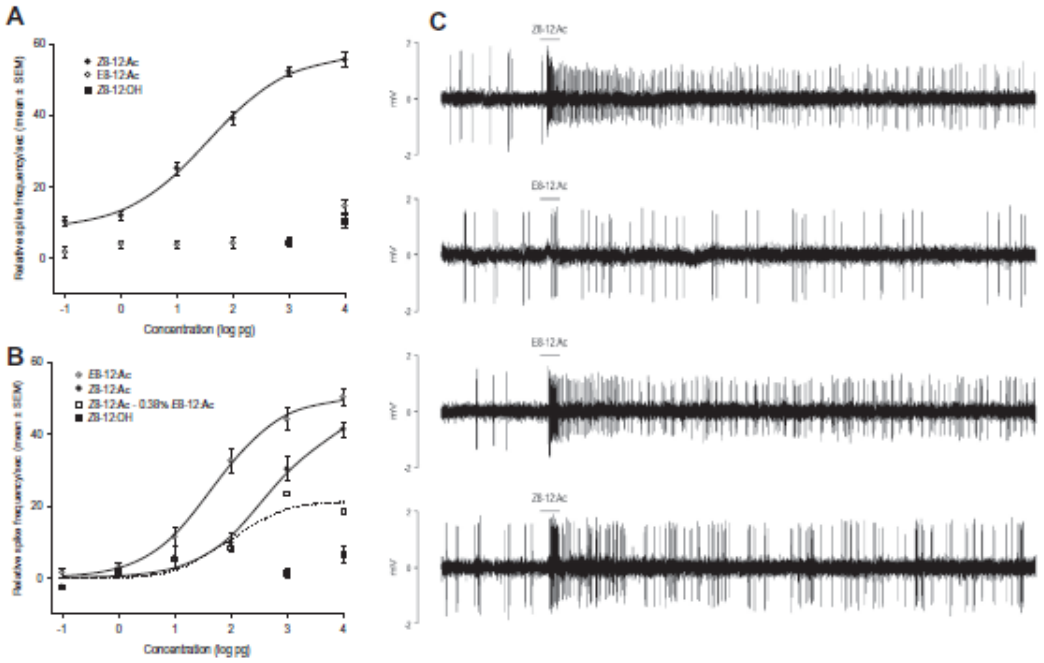

Figure 2. Response of pheromone ORNs of *G. molesta* males to 200 ms puffs of each of the three pheromone components at several concentrations. Dots show observed data (mean ± SEM) and lines the adjusted curves. A) *Z*8-12:Ac ORNs responded strongly to *Z*8-12:Ac and minimally to *E*8-12:Ac and *Z*8-12:OH. The response to *Z*8-12:Ac had sigmoidal shape in the log10 dose scale and was modelled with a non-linear regression (spike frequency = 8.17+(57.42-8.17)/(1+exp((1.55-log10(pg))/0.73), r2=0.99), where "pg" is the loading quantity of the stimulus in pg. N=33 for *Z*8-12:Ac, *Z*8-12:OH and for 1 and 10 ng of *E*8-12:Ac. For the other concentrations of E8-12:Ac, N=4-16. Average response to hexane = 6.4 ± 1.2 spikes/s (mean ± SEM). B) *E*8-12:Ac ORNs responded strongly to *E*8-12:Ac, less intensely to Z8-12:Ac, and almost no response to *Z*8-12:OH was observed, which was tested only at the two highest concentrations. N=10-14 for all compounds. The response to E8-12:Ac was modelled (spike frequency = 50.57/(1+exp((1.69-log10(pg))/0.59)), r2= 0.99) and this equation was used to subtract the contribution of the 0.38% *E*8-12:Ac in *Z*8-12:Ac from the response of *E*8-12:Ac ORNs to *Z*8-12:Ac. The equations describing the response to Z8-12:Ac before and after correction are, respectively, spike frequency = 46.462/(1+exp((2.67-log10(pg))/0.61)), r2= 0.99, and spike frequency = 21.47/(1+exp((1.98-log10(pg1))/0.46)), r2= 0.90, where "pg1" refers to the 0.38% E8-12:Ac in the amount of Z8-12:Ac shown in the x-axis. Average response to hexane = 0.14 ± 1.13 spikes/s (mean ± SEM). C) Representative recordings of one *Z*8-12:Ac ORN (top) and one *E*8-12:Ac ORN (bottom) stimulated with 1ng of *Z*8-12:Ac or *E*8-12:Ac (200 ms puffs: horizontal bar over the trace).

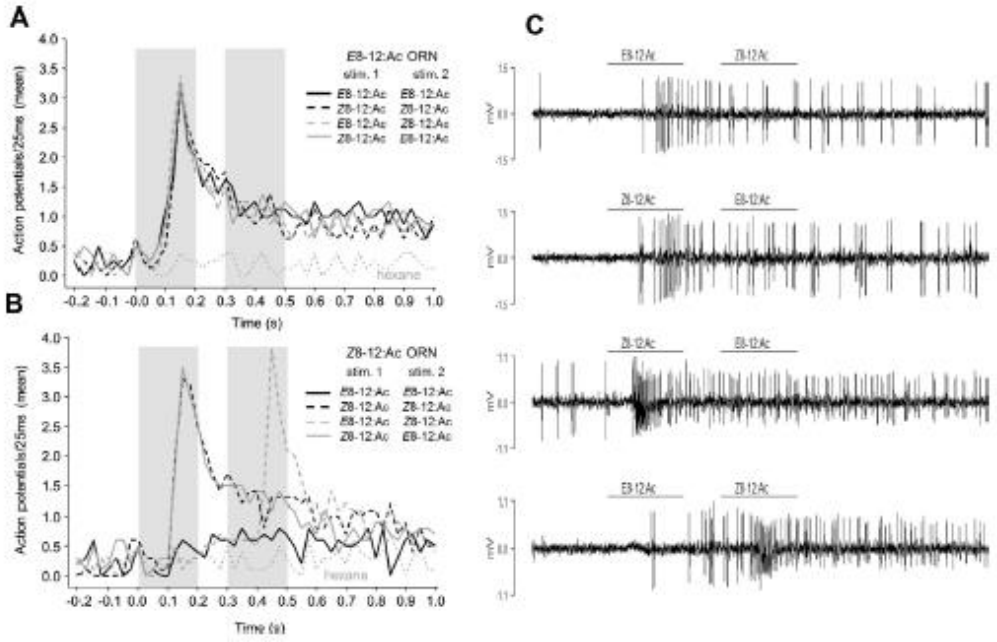

Figure 3. Cross-adaptation test in *E*8-12:Ac ORNs (A, N=8) and *Z*8-12:Ac ORNs (B, N=10) in *G. molesta* males. ORNs were stimulated with two closely spaced puffs of *Z*8-12:Ac or *E*8-12:Ac (grey bars). The relative frequency of spikes in 25ms bins is plotted against time. Notice the lack of response to the second stimulation of the opposite stimulus in cross-stimulus tests with *E*8-12:Ac ORNs (A), indicating that the same cell responds to both compounds. By contrast, in the *Z*8-12:Ac ORNs (B) stimulation with *Z*8-12:Ac adapts the cell to *Z*8-12:Ac but stimulation with *E*8-12:Ac does not, as is expected in highly specific ORNs. The loading quantities of *Z*8-12:Ac and *E*8-12:Ac were 100 pg and 1 ng, respectively, in both ORN types. Only means are shown for the sake of clarity. C) Representative traces of the cross-stimulations (two top traces: one *E*8-12:Ac ORN; two bottom traces: one *Z*8-12:Ac ORN). Each horizontal bar represents a 200 ms puff of either *Z*8-12:Ac or *E*8-12:Ac.

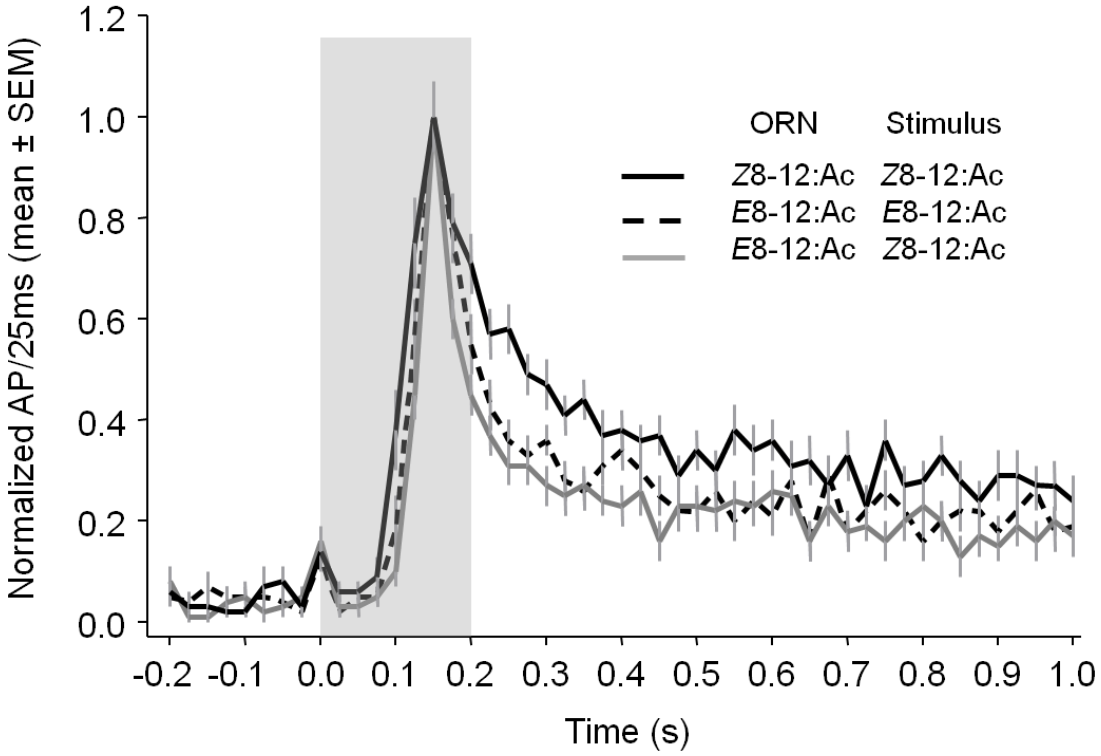

Figure 4. Response dynamics of Z8-12:Ac and E8-12:Ac ORNs to stimulation with Z8-12:Ac (100pg in Z8-12:Ac ORNs and 1ng in E8-12:Ac ORNs), and of E-ORNs to stimulation with 100 pg of E8-12:Ac (N=27-35). Stimulation with Z8-12:Ac resulted in a significantly longer-lasting response in Z8-12:Ac ORNs than in E8-12:Ac ORNs (t-test, $P < 0.05$). E8-12:Ac ORNs displayed similar dynamics to the two pheromone compounds.

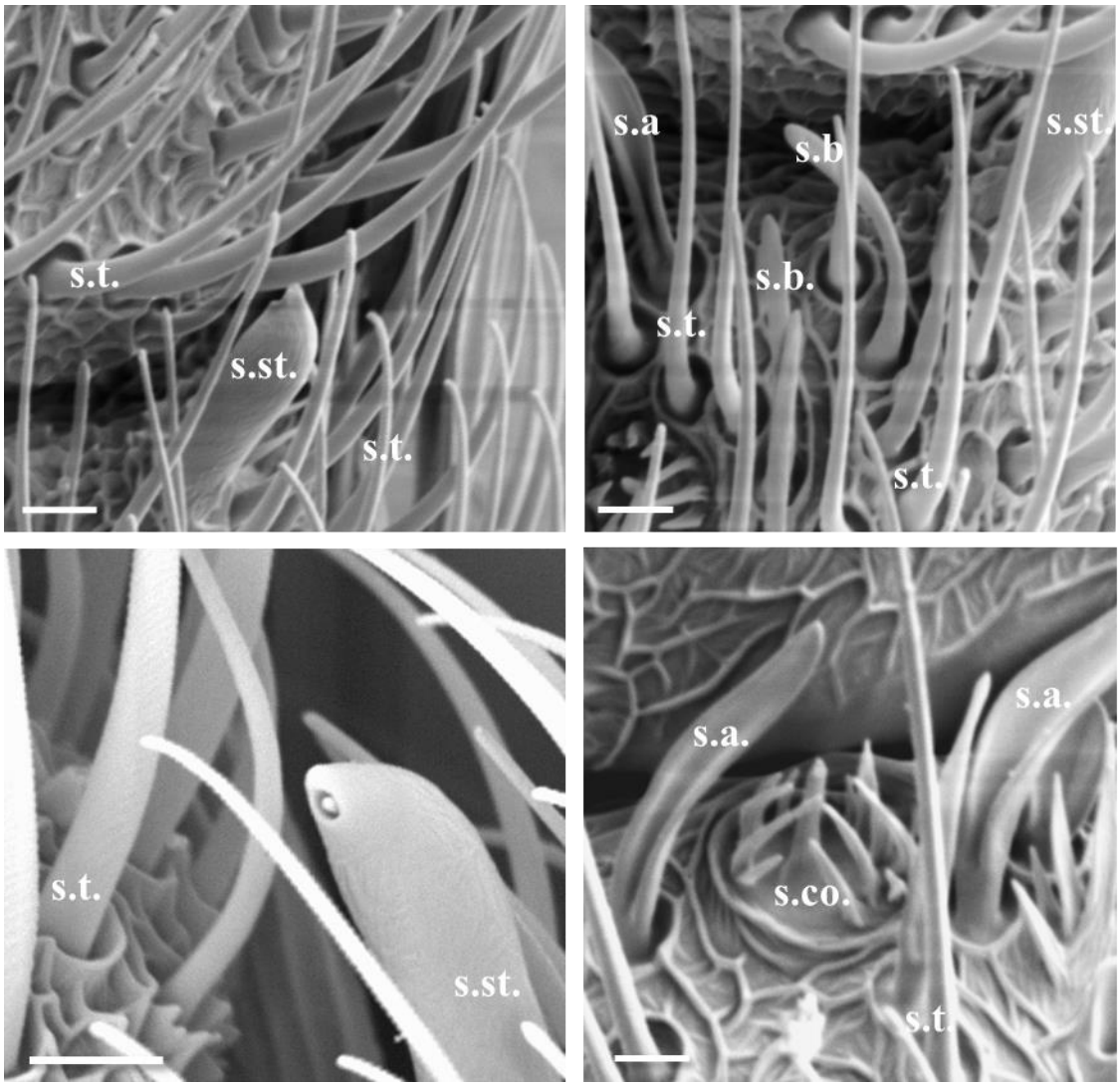

Figure S1. Different types of sensilla on the antennae of male *G. molesta*. Sensilla auricillica (s.a), sensilla basiconica (s.b), sensilla coeloconica (s.co), sensilla styloconica (s.st.), and sensilla trichodea (s.t). Scale bar = 5 µm.

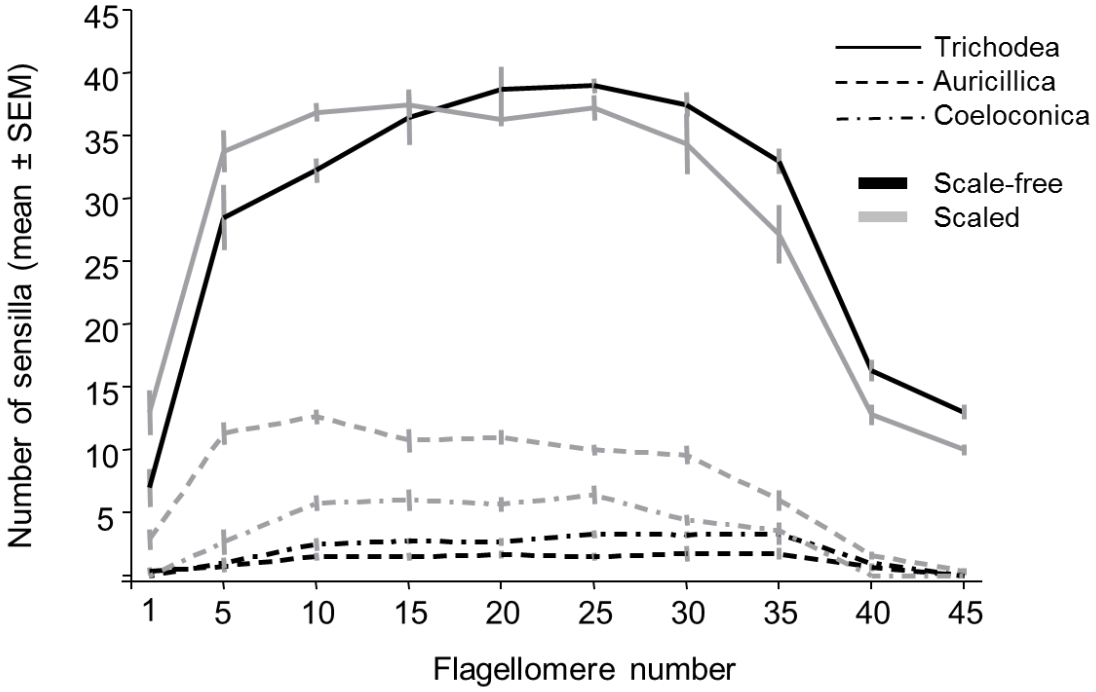

Figure S2. Abundance of three sensilla types (trichodea, auricillica and coeloconica) along the antennal flagellum of *G. molesta* males.

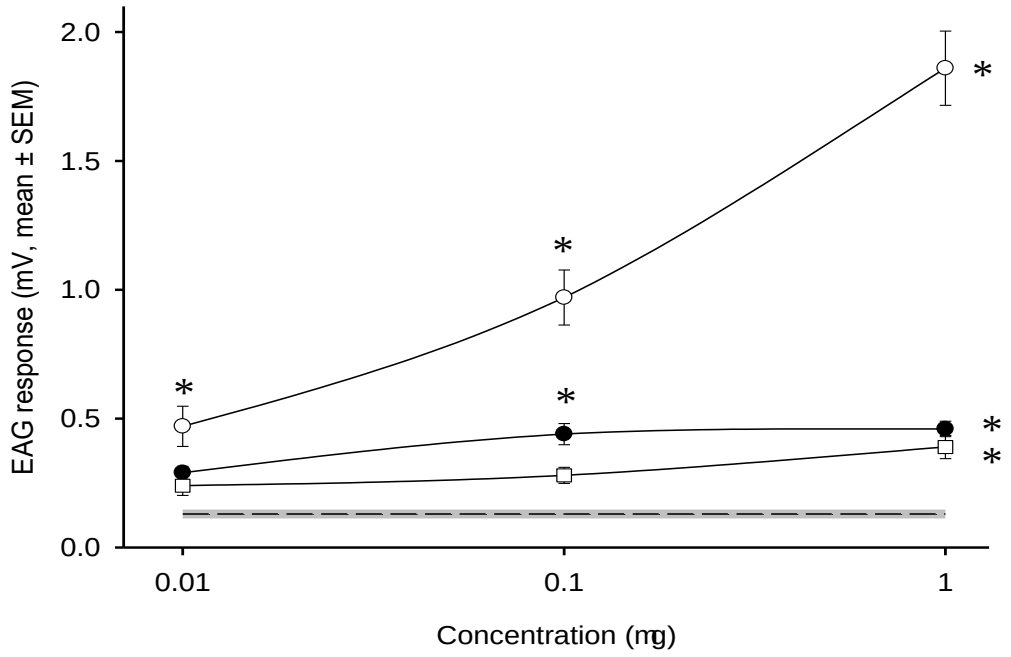

Figure S3. Electroantennographic responses of male *G. molesta* antennae to the three pheromone components. Asterisk indicates difference with hexane within a concentration (GLM followed by planned contrasts, $P < 0.05$)

CHAPTER IV

Interference of plant volatiles on pheromone receptor neurons of male *Grapholita molesta* (Lepidoptera: Tortricidae)

Interference of plant volatiles on pheromone receptor neurons of male *Grapholita molesta* (Lepidoptera: Tortricidae)

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Abstract

In moths, sex pheromone components are detected by pheromone-specific olfactory receptor neurons (ph-ORNs) housed in sensilla trichodea in the male antennae. In Grapholita molesta, ph-ORNs are highly sensitive and specific to the individual sex pheromone components, and thus help in the detection and discrimination of the unique conspecific pheromone blend. Plant odours interspersed with a sub-optimal pheromone dose are reported to increase male moth attraction. To determine if the behavioural synergism of pheromone and plant odours starts at the ph-ORN level, single sensillum recordings were performed on Z8-12:Ac and E8-12:Ac ph-ORNs (Z-ORNs and E-ORNs, respectively) stimulated with pheromone-plant volatile mixtures. First, biologically meaningful plant-volatile doses were determined by recording the response of plant-specific ORNs housed in sensilla auricillica and trichodea to several plant odorants. This exploration provided a first glance at plant ORNs in this species. Then, using these plant volatile doses, we found that the spontaneous activity of ph-ORNs was not affected by the stimulation with plant volatiles, but that a binary mixture of sex pheromone and plant odorants resulted in a small (about 15 %), dose-independent, but statistically significant, reduction in the spike frequency of Z-ORNs with respect to stimulation with Z8-12:Ac alone. The response of E-ORNs to a combination of E8-12:Ac and plant volatiles was not different from E8-12:Ac alone. We argue that the small inhibition of Z-ORNs caused by physiologically realistic plant volatile doses is probably not fully responsible for the observed behavioural synergism of pheromone and plant odors.

Key words: Single sensillum recording, olfactory receptor neuron, plant volatiles, sex pheromone

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1. Introduction

Semiochemicals play an important role in insect communication (Bruce and Pickett, 2011; Beyaert and Hilker, 2014). Male moths follow the pheromone plume trails emitted by conspecific females for mating (McNeil, 1991; Landolt and Phillips, 1997). Moreover, male and female moths are attracted to host plant volatiles (Bruce and Pickett, 2011) derived from a large variety of secondary metabolites (Pichersky and Gershenzon, 2002). In addition to pheromone cues, males also use host plant cues to find females to mate, since females choose suitable host plants to lay eggs (Landolt and Phillips, 1997). For successful mate and host location it is crucial to detect the right proportion of individual components (i.e., odorants) in the volatile blend (i.e., odour) (Bruce and Pickett, 2011; Baker et al., 2012). The simultaneous presence of pheromone and plant odours could either help locating a mate, mask the female pheromone, or be neutral, without any effect on the female emitted pheromone (Deisig et al., 2014). There is evidence that the behavioural response of males to sex pheromone is increased by host plant volatiles (Reddy and Guerrero, 2004). Currently, efforts are dedicated to investigate the potential use of pheromones and other semiochemicals in pest management (Szendrei and Rodriguez-Saona, 2010; Witzgall et al., 2010).

In the last decade several studies have aimed to understand how the mixture of pheromone and plant odorants is reported by olfactory receptor neurons (ORNs) to higher processing centers in the brain, such as the antennal lobe (AL) (De Bruyne and Baker, 2008; Deisig et al., 2014). In moths, pheromone components are detected by highly specialised ORNs housed in sensilla trichodea, and all pheromone-specific neurons converge in the macroglomerular complex (MGC) of the AL (Hansson and Anton, 2000; Baker et al., 2012). Both generalist and specialist ORNs housed in different sensilla types are involved in the detection of general odorants, including plant volatiles (Andersson et al., 1995; 1996; Ansebo et al., 2005, Deisig et al., 2012; Binyameen et al., 2012), and converge in many ordinary glomeruli (OG) in the AL (Hillier and Vickers, 2007; Deisig et al., 2014). Integration of pheromone and plant odours takes place in the AL, however there is evidence that odours also interact at the peripheral receptor level in pheromone-specific ORNs. For example, in *Helicoverpa (=Heliothis) zea* (Boddie), stimulation with binary mixtures of the major pheromone component, (*Z*)-11-hexadenal, and increasing dosages of either linalool or (*Z*)-3-hexenyl acetate, significantly synergise ph-ORNs firing rate compared with responses to the major pheromone component alone (Ochieng et al., 2002). By contrast, electrophysiological studies on ph-ORNs of *Heliothis virescens* (Fabricius) (Hillier and Vickers, 2011), *Spodoptera littoralis* (Boisduval) (Party et al., 2009) and *Agrotis ipsilon* (Hufnagel) (Deisig et al., 2012) have found that firing activity to pheromone is suppressed when plant odorants are co-applied. So, both excitation and inhibition to mixtures of pheromone and plant stimuli are observed at the peripheral level.

The oriental fruit moth, *Grapholita molesta* (Busck), is an oligophagous pest of stone and pome fruits. Larvae bore to new growth shoots and cause economic damage (Rothschild and Vickers, 1991). Female *G. molesta* emit a three-component blend of (*Z*)-8 dodecenyl acetate (*Z*8-12:Ac), (*E*)-8 dodecenyl acetate (*E*8-12:Ac), and (*Z*)-8 dodecenyl alcohol (*Z*8-12:OH), at a ratio of 100:6:10, respectively. A synthetic mixture of the blend

is used in pest management (Roelofs et al., 1969; Linn and Roelofs, 1983; Kong et al., 2014). Field studies report that male and female *G. molesta* are attracted to host-plant volatile blends (Il'ichev et al., 2009; Lu et al., 2012, 2014), and that terpinyl acetate (Knight et al., 2014) and (*Z*)-3 hexenyl acetate (Yu et al., 2014) increase male captures in pheromone traps. A 5-component plant odour blend behaviourally attractive to female *G. molesta* (Piñero and Dorn, 2007) synergises male response to a sub-optimal pheromone dose in the wind tunnel (Varela et al., 2011a), and addition of citral to *Z*8-12:Ac increases electroantennogram (EAG) responses compared to *Z*8-12:Ac alone (Faraone et al., 2013).

In this study, we explore whether physiological changes at the peripheral receptor level could explain the behavioural synergism produced by the mixture of pheromone and plant odors in male *G. molesta* (Varela et al., 2011a). We made single sensillum recordings from *Z*8-12:Ac and *E*8-12:Ac specific-ORNs (Ammagarahalli and Gemeno, 2014) stimulated with sex pheromone and plant volatiles independently or mixed in a blend, to determine if the response of these ORNs to sex pheromone is altered by co-stimulation with plant volatiles. We tested three plant blends with reported behavioural activity (Piñero and Dorn, 2007; Il'ichev et al., 2009; Lu et al., 2012), individual components from each blend, and additional odorants that could be biologically relevant. We tested them at several doses to account for possible concentration effects. Plant volatiles were tested first in non-pheromone ORNs from sensilla auricillica and trichodea to characterize the response of these yet unexplored ORNs, and to determine biologically-relevant plant doses to be used in the pheromone-plant interaction test.

2. Materials and methods

2.1. Insects

The colony of *G. molesta* was established at the University of Lleida, Spain, in 2005 with individuals formerly collected in peach orchards and reared in laboratory in Piacenza, Italy. Larvae were reared on a semi-synthetic diet modified from Ivaldi-Sender (1974) under a L16:D8 photoperiod at 25 ± 1 ºC. Male pupae were placed in 4-l polypropylene containers provided with a cotton ball soaked in 10 % sucrose solution. Adult emergence was checked daily and adults were used when 2-4 days old. Care was taken not to expose adults to synthetic odours before the tests.

2.2. Odorant stimuli

Individual volatile compounds (i.e., odorants) and their blends (i.e., odours), were tested in the study (Table 1, chemical details in Table S1). The "Chinese" plant blend was identified from pear fruit volatile collections and it attracts males and females in the field and in the laboratory (Lu et al., 2012). The "Swiss" blend was identified from peach shoot volatiles and it attracts mated females in the laboratory (Piñero and Dorn, 2007) and synergizes male response to a suboptimal pheromone dose in the laboratory (Varela et al., 2011a). Finally, the "Australian" blend, which was identified from peach shoot volatiles, but has a different composition than the Swiss blend, attracts males in the field (Il'ichev et al., 2009). We prepared three plant blends emulating the "Australian",

"Chinese", and "Swiss" blends (Table 1). Selected compounds from these blends, and others that have shown behavioural or electrophysiological activity in *G. molesta* (Faraone et al., 2013; Knight et al., 2014), or that are released by peach or apple plants (Natale et al., 2003, Casado et al., 2006, Wang et al., 2009, Lu et al., 2012), were tested individually (Table 1).

Sex pheromone compounds were provided by Pherobank (The Netherlands) with an initial purity ≥99 %. Gas chromatographic analysis revealed that *Z*8-12:Ac contained 0.38 % *E*8-12:Ac, and that *E*8-12:Ac contained 0.24 % *Z*8-12:Ac. Pure pheromone and plant compounds were weighed and diluted in *n*-hexane to prepare 10 μg/µl stock solutions. The pheromone blend consists of a mixture of *Z*8-12:Ac, *E*8-12:Ac and *Z*8-12:OH in a 100:6:10 ratio.

2.3. Electrophysiological recordings

Males were immobilized using CO_2 for 10 s and were mounted on a handcrafted poly(methyl methacrylate) insect holder. The insect was inserted through a vertical hole drilled in the holder´s body and the protruding head was restrained by fixing a piece of adhesive cloth tape between the head and the holder`s surface. The antennae were carefully laid on a slant surface attached to the holder´s top that was lined with double sided sticky tape, and were oriented for easy access with the electrodes. To record from sensilla located on the scaled area (sensilla trichodea or auricillica), scales were removed by gently rolling the antennae on the sticky tape, and the remaining scales were removed individually with the help of a tungsten electrode. Sub-millimetric smoking paper strips placed over the antennae and glued on the sticky surface prevented antennal torsion. A stereo microscope (objective 2x, oculars 25x, zoom range 0.8-12.5, Leica Microsystems, Madrid, Spain) was used in performing these operations and to visualize the recordings. These were obtained by means of electrolytically (saturated KNO_2) sharpened tungsten microelectrodes (0.125-mm diameter, 99.98 % purity, Advent Research Materials Ltd, England). The reference electrode was inserted in the head through the mouth parts. The recording electrode was situated near the base of a randomly chosen sensillum and pushed gently inward with the help of a manual micromanipulator (NMN-25, Narishige, Japan) until spikes were detected. Flagellomeres 10-35 were sampled. Recordings from sensilla auricillica were made in the distal scaled area, and those from sensilla trichodea were distributed randomly in the scaled and scale-free areas. The signal from the recording electrode was pre-amplified (10x gain, Universal Single Ended Probe, Syntech, Germany), filtered (1 KHz and 300 Hz for high and low cut-off filters, respectively), digitized (IDAC-4, Syntech, Germany), and analyzed in a PC (AutoSpike v.3.9, Syntech, Germany). Sampling rate of the recording wave signal was 10666.7 samples s^{-1}. The setup was mounted on an anti-vibration table (63-511, TMC Ametek, USA) and was shielded by a Faraday cage to reduce low frequency noise.

2.4. Odour stimulation

Dilutions were applied as 1 μl aliquots (1 μl micropipettes, Drummond Scientific Co., USA) on 1 x 20 mm *n*-hexane-pre-cleaned filter paper strips (#1, Whatman International Ltd, England). After having dried (5 min) the filter papers were introduced

in *n*-hexane-pre-cleaned 100 µl glass micropipettes (1.2 mm internal diameter, Blaubrand® Intramark, Germany) which were then stored in glass test tubes sealed with PTFE-coated screw caps until used. New stimuli cartridges were prepared each day, and a given stimulus cartridge was not used for more than 10 stimulations. Air flow was generated by two diaphragm aquarium pumps connected to a 3-way solenoid valve (CS-55, Syntech, Germany). A 0.5-l/min flow of charcoal-filtered and humidified air blew continuously over the insect preparation through a 5-mm internal diameter plastic tube placed 15-20 mm from the preparation (air velocity at exit = 0.4 m/s). A stimulus cartridge was attached to the puff-flow with the side bearing the filter paper positioned 0.4 cm down from the recording point and perpendicular to the direction of the continuous air flow. A 0.2-l/m charcoal-filtered room air flow was puffed through the odour cartridge towards the recording spot for 200 ms (air velocity at exit = 2.9 m/s). The flow of continuous humid air was decreased by 0.2-l/min during the puff. Time interval between puffs was at least 60 s, but longer if needed to let the spike activity return to pre-stimulation levels. A maximum of 4 sensilla were recorded per insect, and at least a 30 min interval between two sensilla recordings was allowed. The air around the preparation was constantly renewed with an exhaust to minimize contamination. Test tubes for keeping stimulus pipettes were rinsed with acetone and heated at 250 ºC overnight before being reused.

2.5. Response of non-pheromone specific ORNs to plant odorants

In order to choose biologically relevant plant doses for testing the response of pheromone-specific ORNs to pheromone and plant volatiles, we first determined the response of ORNs housed in sensilla auricillica, and of non-pheromone ORNs housed in sensilla trichodea, both of which are the most likely receptor neurons of plant volatiles (Ansebo et al., 2005; Binyameen et al., 2012), to several plant volatiles at several doses. In *G. molesta* most of the sensilla trichodea (64 %) house one ORN responding only to *Z*8-12:Ac (*Z*-ORNs), 7 % house one ORN responding strongly to *E*8-12:Ac (*E*-ORNs) and weakly to *Z*8-12:Ac, and 29 % have ORNs that do not respond to the pheromone compounds (Ammagarahalli and Gemeno, 2014). Sensilla trichodea distribute evenly throughout the flagellum´s surface, whereas sensilla auricillica, which are readily distinguishable for their flattened shape and small size, occur mainly on the distal edge of the flagellomere and are more abundant in the scaled area (Ammagarahalli and Gemeno, 2014). Olfactory neurons in sensilla trichodea were first stimulated with individual sex pheromone compounds (1 ng of each, *Z*8-12:Ac, *E*8-12:Ac, *Z*8-12:OH) to determine if they were pheromone-specialist. Non pheromone-specific ORNs were further stimulated with 0.01 µg of the pheromone blend and 0.1 µg of several biologically relevant plant odorants, randomising the order of stimuli among ORNs. Preliminary tests showed that sensilla auricillica do not house pheromone specific ORNs, so they were stimulated with individual plant odorants (0.1 µg), with each of the 3 individual pheromone components (0.01 µg) and with the pheromone blend (0.01 µg). The order of stimuli was *n*-hexane, followed by the pheromone blend and its components, and the plant odorants in random order. ORNs from both sensilla types with relatively strong responses

to a given plant compound were further stimulated with increasing doses (0.001 to 10 μg) of the most sensitive compounds to obtain dose-response curves.

2.6. Response of pheromone-specific ORNs to pheromone and plant odorants

Experiments were carried out to test whether individual plant compounds or plant blends (Swiss, Chinese, and Australian) (Table 1), affect the response of pheromone-specific ORNs to their sex pheromone ligands. Dose-response curves from plant-ORNs showed that a physiologically-relevant range of plant odorant doses was 10 to 100 ng. A fixed concentration of sex pheromone (*Z*8-12:Ac or *E*8-12:Ac, 0.1 ng) was mixed with increasing concentrations of plant volatiles to make 1:0, 1:1, 1:10, 1:100 and 1:1000 pheromone:plant blends. Plant blends were tested in *Z*- and *E*-ORNs but plant odorants were not tested in *E*-ORNs due to their sparse number and distribution. Solvent (1 μl on filter paper) and individual plant volatiles or plant blends (100 ng) were control treatments. To make the pheromone:plant blends, 100 µl of a 1 ng/μl pheromone stock solution (*Z*8-12:Ac or *E*8-12:Ac) was added to a 2 ml vial, and the volume was completed to 1 ml by adding *n*-hexane and different volumes of 1 ng/μl, 100 ng/μl and 10 µg/μl plant volatile solutions. Pheromone and pheromone plus plant solutions were prepared on the same day using the same pheromone stock solution, and therefore all of them contained the same pheromone concentration.

ORNs were first stimulated with 0.1 ng of *Z*- or *E*8-12:Ac to determine their ligand specificity. Once pheromone specificity was determined, the ph-ORNs were stimulated with the same treatment order: hexane, sex pheromone ligand (*Z*8-12:Ac or *E*8-12:Ac, 0.1 ng), plant volatiles (odorants or blends, 100 ng), pheromone:plant blends with a constant pheromone dose (0.1 ng) and increasing doses of the plant volatile (1:1, 1:10, 1:100 and 1:1000), and a second 0.1 ng *Z*- or *E*8-12:Ac puff at the end of the treatment run to control for possible neuron adaptation.

2.7 Spike and statistical analyses

When more than one spike size was detected, they were sorted by their shape and amplitude. ORNs were labelled large, medium and small (L, M and S, respectively) according to their relative size in each sensillum. For each puff, the number of spikes during a 1-s pre-stimulation period was subtracted from the number of spikes during a 1-s post-stimulation period to obtain the relative number of spikes per second, and this variable was analysed statistically. To determine if plant volatiles affect the response of pheromone-specific ORNs to sex pheromone, we used a model in which the difference in spikes before and after the puff was a function of the pheromone:plant dose and the plant volatile composition. The effect of plant blends on *Z*- and *E*-ORNs was analysed separately. Since the data were not normally distributed we run generalized linear models (GLM) in R (Bolker et al., 2009; R Core Team, 2013). Multiple pair-wise comparisons among treatment means were performed with least-squares means method using the "lsmeans" and "mcp" packages of R (R Core Team, 2013). Raw data, R codes and R outputs are available as supplementary material.

3. Results

3.1. Response of non-pheromone specific ORNs to plant odorants

Both excitation and inhibition were observed, but in general, responses were very similar to solvent stimulation, and very few ORNs showed any specialization. The number of ORNs per sensilla varied from one to three, with distinguishable large, medium, and small spike amplitudes. In most cases, only one neuron per sensillum was clearly excited by the plant odorants.

3.1.1. Sensilla trichodea

Sixty-four ORNs housed in 25 sensilla trichodea of 12 individuals were tested with 3 to 10 plant odorants, sex pheromone and *n*-hexane. Most of the sensilla (66 %) housed 3 ORNs (large, medium and small amplitude), whereas the remaining 34 % housed 2 ORNs (large and small amplitude). The spontaneous activity of large amplitude ORNs was <5 spikes s^{-1}, whereas that of medium and small amplitude ORNs was between 19 and 33 spikes s^{-1} (Table S2). Stimulation with plant odorants elicited between -36 and 79 spikes s^{-1} (Fig. 1A, ORNs 14S and 8S, respectively). Most ORN responses to plant volatiles were very similar to the response elicited by *n*-hexane (HEX, yellow colour range in raster plot, Fig. 1A). Specific response to one or a few plant odorants was rare, but a group of four small-amplitude ORNs (6 % of all the ORNs) produced relatively high spike counts to stimulation with farnesene (FAR) (Fig. 1A, ORNs 3S, 8S, 9S, 18S), while they showed strong inhibition to the other compounds (Fig. 1A, ORNs 8S, 9S). Spontaneous activity of neurons co-localized in the same sensilla as the FAR-specific ORNs was practically unchanged by plant odorant stimulation (Fig. 1A, ORNs 3L, 3M, 8L, 9L, 18L). The spiking response of FAR-ORN 8S is illustrated in Fig. 2A. Few other ORNs in sensilla trichodea showed some degree of specialization. Benzaldehyde (BZA) inhibited ORN 14S and excited ORN 12S, pear ester (PE) excited ORN 4S, terpinyl acetate (TA) excited ORN 15S, and (*Z*)-3-hexenyl acetate (Z3HA) excited ORN 10S. The 6 ORNs housed in sensilla trichodea 24 and 25, were stimulated with 10 additional compounds but did not show specificity (data not shown). Only one ORN (6S) showed some response to sex pheromone (PHE).

Dose-response curves of sensilla trichodea ORNs were only made for three of the four FAR-specific neurons. All of them showed a typical sigmoidal-shape response in the log-dose scale, with little excitation to 1 and 10 ng doses and a sharp increase in the response to doses from 10 ng to 10 µg (Fig. 3A, Fig. 2A). *n*-hexane stimulation produced minute changes in spontaneous activity (Fig. 1A, Fig. 3A).

3.1.2. Sensilla auricillica

Eighty eight ORNs from 40 sensilla auricillica from 20 males were tested with 5 to 10 plant odorants, sex pheromone and *n*-hexane (Fig. 1B). Another 60 ORNs from 20 sensilla from 8 males were tested with 18-20 plant odorants, the pheromone blend and its individual components and *n*-hexane (Fig. 1B). More than half (67 %) of all the sensilla auricillica housed 3 ORNs, whereas a smaller percentage (29 %) housed two neurons, and the remaining 3 % sensilla housed a single neuron. The spontaneous activity of large

spike-amplitude ORNs was between 1 and 2 spikes s^{-1} in sensilla housing 2 or 3 neurons, but it rose to 26 spikes s^{-1} in sensilla housing just one ORN (Table S2). Medium and small spike-amplitude ORNs had between 24 and 34 spikes s^{-1} (Table S2). Fig. 2B shows the response of an auricillic sensillum (52 in Fig. 1B) whose large ORN responded to methyl salicylate (MS), the medium one to 1-octen-3-ol (3OH), whereas the small one was silent.

Stimulation with plant odorants elicited between -35 (ORN 53L) and 97 (ORN 4L) spikes s^{-1}, but most ORN responses were very similar to the response elicited by *n*-hexane (yellow range colour, Fig. 1B), and very little specialization was observed. Some ORNs showed moderate to high excitation to most of the plant odorants tested, including pheromone (2S, 3S, 5L, 14S, 19L, 21M, 31L, 33S, 36S, 37M, 41M, and 58M), but these were cells that normally showed relatively high responses to *n*-hexane. A few ORNs were broadly inhibited (9L, 23S and 32M) (Fig. 1B). Strong excitation and inhibition to different compounds for the same ORN was observed in several ORNs (e.g., 1L, 4L, and 53L). Out of the 60 ORNs stimulated with 18-20 plant odorants, two of them (52L, 53L) were relatively specific to methyl salicylate (MS) whereas a third one (52M) showed specificity to 1-octen-3-ol (3OH). ORN 32M was inhibited by most compounds but excited by benzonitrile (BZN), and cell 22S was excited by the pear ester (PE).

Dose-response curves were obtained for 5 plant odorants on 9 different ORNs (Fig. 3B-F). Except for the benzaldehyde ORN (Fig. 3F), for the rest of the plant odorants there was a typical dose-response pattern, where at low doses the ORNs were little excited, but at higher doses they were very responsive. Some ORNs (BZN 3S, and TA 37M and 37S, Fig. 3B and C) were not affected by the increase in plant odorant dose, but these cells were not very specific to these compounds (Fig. 1B). The level of response of the auricillic ORNs was similar to that of the trichodea FAR ORN, with the 100 ng dose producing about 50 spikes s^{-1}. *n*-hexane stimulation produced minute changes in spontaneous activity in ORNs from sensilla auricillica (Fig. 1B, Fig. 3).

3.2. Response of pheromone-specific ORNs to mixtures of pheromone components and plant volatiles

Recordings were obtained from 112 *Z*-ORNs and 15 *E*-ORNs from 34 and 7 male moths, respectively. The percentage of sensilla with one, two, or three neurons was 60 %, 31 % and 9 %, respectively in *Z*-ORNs, and 73 %, 27 % and 0 %, respectively in *E*-ORNs. ORNs co-localized with pheromone ORNs did not respond to stimulation with sex pheromone or plant volatiles. *Z*- and *E*-ORNs produced less than 3 spikes s^{-1} upon stimulation with plant volatiles or *n*-hexane (Fig. 4B, and Fig. 5B and C), however they were clearly excited (about 40 spikes s^{-1}) by a medium dose (0.1 ng, Ammagarahalli and Gemeno, 2014) of their respective pheromone ligands (Fig. 4A and 5A).

Although Z-ORNs did not respond to plant stimuli alone, the addition of plant *odorants* or plant *blends* to the sex pheromone resulted in a relatively small (about 15 %) but statistically significant reduction in the response of *Z*-ORNs to *Z*8-12:Ac (Figs. 4 and 5, A and B) ($df = 7$, $F = 316$, $P<0.001$, for plant odorants; $df = 7$, $F = 88$, $P<0.001$ for plant blends). The reduction of *Z*-ORN response to pheromone was independent of the amount of plant odorant present in the mix, *i.e.*, all the plant odorant doses reduced the response and higher plant volatile doses did not result in further reduction (Fig. 4B). The

reduction of plant blends on *Z*-ORN pheromone response was only at the 1:1 and 1:1000 pheromone:plant ratios, and only with respect to the second pheromone puff, with no clear dose-response trend (Fig. 5B). Odorants differed in the reduction that they elicited in Z-ORNs (df=10, F=6.94, P<0.001) with BZA and TA producing less spikes than BZN, CIT and EB (Fig. 4C). When analysed individually, only 4 plant odorants decreased responses to the first pheromone puff, and they did so at the pheromone:plant ratios 1:1(E2A and TA), 1:10 (TA, Z3OH), 1:100 (TA), and 1:1000 (CIT) (Tukey´s test, P < 0.05). One compound (TA) also reduced the response to the second pheromone puff, and it was at the 1:1 ratio (Tukey´s test, P < 0.05). Plant blends differed in their effect on Z-ORN (df=2, F=9.08, P<0.001), where the Chinese blend produced significantly lower responses than the other two blends (Tukey´s test P<0.001). There was no significant interaction in *Z*-ORNs between plant doses and plant odorants (df = 70, F = 0.95, P = 0.6) or plant blends (df=14, F=0.81, P=0.65). Fig. 2C illustrates the response of a *Z*-ORN to pheromone, TA, and the blend of the two stimuli.

E-ORNs did not respond to hexane or the plant blends, and their response to *E*8-12:Ac was not affected by the addition of the plant blends (Fig. 5A and C, df=7, F=94.18, P<0.001).

4. Discussion

4.1 Plant-specific ORNs

In a previous study, we found that 29 % of the sensilla trichodea of male *G. molesta* contained ORNs that did not respond to the female sex pheromone components (Ammagarahalli and Gemeno, 2014). The present study reveals that some of these pheromone-unresponsive ORNs may be tuned to plant-odorants. In general, the response of sensilla trichodea and sensilla auricillica ORNs to plant volatiles was not very different from the response to solvent stimulation, and a large percentage (probably more than 50 %, depending on the threshold criteria used) of these ORNs could be considered unresponsive to the stimuli panel that we have tested. Other moth studies report similar percentages of unresponsive ORNs in either sensilla trichodea [e.g., 71 % in *Trichoplusia ni* (Hübner) (Todd and Baker, 1993), 51 % in *S. littoralis* (Jönsson and Anderson, 1999), 22 % in *Manduca sexta* (L.) (Shields and Hildebrand, 2001)], or sensilla auricillica [60 % in *Cydia pomonella* (L.) (Ansebo et al., 2005)], so it appears that non-responding ORNs are not uncommon, but the reasons for such widespread ORNs "silence" are still unclear.

One possible explanation for the high number of unresponsive ORNs is that many plant ORNs have narrow molecular receptive ranges (MRR) (i.e., they are specialist), and that if the odour panel with which they are stimulated is modest (like the one we have tested), then only a few of them will show specific responses. Specialist plant ORNs are, indeed, common in moths (De Bruyne and Baker, 2008; Andersson et al., 2015), and in some cases they appear to be relatively abundant, such as the 30 % responding to phenyl acetaldehyde in *S. littoralis* (Binyameen et al., 2012). ORNs in moths are often categorized in response "types" according to their MRR (e.g., Shields and Hildebrand, 2001; Hillier et al., 2006; Binyameen et al., 2012), however in *G. molesta* the only specialist ORN "type" that we could identify was that responding to racemic farnesene

(FAR) in sensilla trichodea. Further specialist responses were found in one or two ORNs, so they were not categorized as types. We explored the presence of further ORN types with statistical group analysis but it did not reveal more distinct ORN types than the already identified FAR ORNs (data not shown). Comparison of dose-response curves between ph-ORNs (Ammagarahalli and Gemeno, 2014) and plant-ORNs (this study) in *G. molesta* reveals that plant-ORNs are at least one order of magnitude less sensitive than pheromone ORNs. To characterize plant ORNs we used a single plant dose of 100 ng, which is within the range of the dose-response curves (1 ng-10 µg). Higher plant doses may have resulted in stronger or more numerous ORN responses, but possibly at the expense of getting a distorted picture if the doses fall outside of the natural-response range (Hallem and Carlson, 2006). In *H. virescens* males the sensitivity of pheromone and plant-ORNs appears to be similar to each other (Hillier and Vickers, 2007), but in *A. ipsilon* males the pheromone ORNs are clearly more sensitive than the heptanal-responding ORNs (Barrozo et al., 2011).

The four FAR-specific ORNs that we describe in here were found only in sensilla trichodea, which suggest that there may be odour-specialization according to sensillum type, as has been observed in other species (Binyameen et al., 2012; Pophof et al., 2005). Interestingly, in two of the FAR ORNs there was a general reduction in spike frequency to most of the other odorants. To some extent this was also observed in the other two FAR ORNs, but we lost contact with them before testing the complete odour panel. Enhanced contrast due to a combination of excitation and inhibition was occasionally observed in other ORNs, mainly in sensilla auricillica. In addition, some of the individual compound responses were inhibitory instead of excitatory. Inhibition, as opposed to excitation, of plant-ORNs is rarely reported in the moth literature that we have examined, and in the few cases where it is reported, it is far less frequent than excitation (Anderson et al, 1995; Pophof et al., 2005). A Concert of excitatory and inhibitory responses to different compounds by the same ORN may increase the coding capability of plant-ORNs and help insects decode a diverse plant stimulus landscape using less ORNs than if only excitatory responses were produced (Bruce and Picket, 2011; Clifford and Riffell, 2013).

Several farnesene isomers occur naturally as constituents of aphid alarm pheromone and apple coating volatiles, among other sources (e.g., Bowers et al., 1977). (*E*)-β-farnesene is an attractant of the tortricid moths *C. pomonella* (Yan et al., 2003), *Lobesia botrana* (Denis & Schiffermüller) (Tasin et al., 2009) and *G. molesta* (Il`ichev et al., 2009; Lu et al., 2012), and it excites ORNs in sensilla auricillica of *C. pomonella* (Ansebo et al., 2005). We tested a racemic farnesene mixture, so we do not know which isomer, or isomers, the FAR ORNs of *G. molesta* are tuned to. Another compound, the pear ester, has been shown to attract male *G. molesta* in dual-choice olfactory tests (Molinari et al., 2010), but other studies suggest that it is not attractive to *G. molesta* males in the field or in the laboratory (personal observation, and Knight and Light, 2004). Correspondingly, the ORN responses to this compound were mild and unspecific. By contrast, the pear ester is important in the behaviour of *C. pomonella*, and a high proportion of the ORNs respond with high specificity to this compound (Ansebo et al., 2005). Another compound that produced relatively specific responses in *G. molesta* was

methyl salicylate. This compound was found in volatile collections of fruits but it was not tested behaviourally with *G. molesta* (Lu et al., 2012). *Mamestra brassicae* (L.) has a very specific ORN for methyl salicylate (Ulland et al., 2008) and this compound attracts *C. pomonella* (El-Sayed et al., 2013); therefore, it could be a potential attractant of *G. molesta*.

Most of the plant volatiles tested were chosen because of their demonstrated behavioural or physiological activity in *G. molesta* males or females (Il`ichev et al., 2009; Lu et al., 2012; Piñero and Dorn, 2007). Therefore, we expected to find more ORN responses than we did. These compounds were identified in non-floral plant parts, but at least 14 of them are also present in flower scents (Knudsen and Tollsten, 1993). Flower odors are probably not very relevant to *G. molesta* males because this species is not known to visit flowers. Possibly, if odours emitted by the natural adult food sources were tested, more ORN responses would be obtained. Surprisingly little is known about the food sources of adult *G. molesta*, although presumably they feed in sugary plant secretions, like the extrafloral nectaries from peach leaves (Atanassov and Shearer, 2005).

4.2 Effect of plant volatiles on pheromone-specific ORNs

We reported previously that the *Z*8-12:Ac and *E*8-12:Ac pheromone-ORNs of *G. molesta* are very specific and sensitive to their natural ligands (Ammagarahalli and Gemeno, 2014). Here we show that ph-ORNs are not sensitive to the plant volatiles. We only tested the highest plant dose (100 ng) with the assumption that if ph-ORNs did not respond to a high plant dose they would not respond to the lower doses either. However, this premise, which is based on the general observation that the ORNs MRR broadens with increased stimulus doses (Hallem and Carlson, 2006), remains to be tested in this particular case. Despite the apparent lack of response of ph-ORNs to plant volatiles, when pheromone and plant stimuli were co-applied, the response of ph-ORNs was lower than when a comparable dose of the pheromone ligand was tested alone. This effect was consistent in the case of *Z*-ORNs tested with plant odorants, but sporadic or absent in the case of *Z*- and *E*-ORNs tested with plant odour blends.

Several characteristics of the pheromone-plant interaction at the ORN level prompt us to speculate that it would have only minor effects on male *G. molesta* behaviour or ecology. First of all, although statistically significant, the reduction in spike frequency was only a modest 15 %, which, according to a calibrated response of ph-ORNs to pheromone doses (Ammagarahalli and Gemeno, 2014), is equivalent to stimulating the ORN with half the pheromone dose, and this would have only a minor effect on male behavioural response, since the number of males that contact an optimal pheromone source is stable over a 100-fold concentration step (Valera et al., 2011a). Secondly, there was no plant-dose effect, i.e., all pheromone:plant blends, from 1:1 to 1:1000, caused similar spiking activity reduction in *Z*-ORNs, which suggests that the suppression is somewhat independent of the plant volatiles themselves and could be due to other causes. For example, a mixture of mineral oil and hexane solutions can affect the release rate of compounds dissolved in each other (Ochieng et al., 2002). Nevertheless, the doses used in our experiment are probably in the same range than what a moth would encounter under natural conditions, as shown by the dose-response curves in the plant-specific ORNs.

Furthermore, the effect of the plant volatiles was not cumulative because ph-ORNs responded similarly to the first sex pheromone puff as to the one following all the pheromone-plant stimulations, indicating that plant stimuli were not causing adaptation. Thirdly, although some plant odorants were slightly more active than others, their effect was fundamentally similar to each other, with a moderate reduction in ORN activity and no marked differences that could allow, *a priori*, sensory discrimination among them by the ph-ORNs. In brief, a modest dose-independent and unspecific decrease in ph-ORN firing rate by several plant odorants, may not, in itself, fully explain the pheromone-plant behavioural synergism that we have previously documented (Varela et al., 2011a).

In other moth species, the effect of plant volatiles on the response of ph-ORNs to pheromone is far more acute than what we report in here. In *A. ipsilon*, a 1:100 blend of pheromone:heptanal reduced responses from about 50 spikes s^{-1} with pheromone alone to about 5 spikes s^{-1} with the pheromone-plant blend, a level comparable to solvent stimulation (Deisig et al., 2012). In *H. virescens*, a 1:1 ratio of pheromone: linalool halved the spiking activity of *Z*11-16:Ac and *Z*11-16:Ald pheromone ORNs (Hillier and Vickers, 2011). In *H. zea*, 1:1 to 1:1000 ratios of *Z*11-16:Ald: linalool almost doubled spike frequency with respect to stimulation with pheromone alone (Ochieng et al., 2002). Furthermore, unlike *G. molesta*, in all these species the effect of the plant volatiles is dose-dependent. It is intriguing, though, that with few exceptions (Ochieng et al., 2002; Hillier and Vickers, 2011), in the majority of species where it has been tested, including *G. molesta*, the effect of plant volatiles is to decrease (and not increase) the response of ph-ORNs to pheromone (Deisig et al., 2014). This is counterintuitive because if pheromone-plant stimuli integration at the ph-ORN level were to explain behavioural synergism, one would expect that the mix would increase, and not decrease, ph-ORN responses to pheromone. A possible physiological function of pheromone suppression by plant volatiles is to improve pheromone pulse resolution, and thus potentially aid male orientation to pheromone-emitting females (Party et al., 2009; Deisig et al., 2014), although this remains to be tested with free-flying insects.

Yet, since plant-specific ORNs are already present on the moth antenna, it seems redundant that moths would also need to dedicate their highly-specialist ph-ORNs (De Bruyne and Baker, 2008) to sense plant volatile stimuli in order to gain additional information about the presence of plant volatiles in the environment. In addition, the available evidence indicates that pheromone and plant stimuli travel *via* separate nerve lines to the AL and that integration takes place in there (Christensen and Hildebrand, 2002; Lei and Vickers, 2008; Namiki et al., 2008), so sensory integration at the peripheral level seems even more redundant. However, in the tortricids *C. pomonella* and *G. molesta* some projection neurons responding to pheromone innervate ordinary glomeruli and not the MGC located at the entrance of the antennal nerve, which typically receives pheromone input from the antenna (Trona et al., 2010, Varela et al., 2011b). This unusual pattern of coding in the AL could be explained by the response of non-pheromone ORNs to both pheromone and plant compounds at the peripheral receptor level in *C. pomonella* (Ansebo et al., 2005), or even in *G. molesta*, as we have shown. The accumulation of cases showing that plant volatiles, or even pheromone compounds (Hillier and Vickers,

2011), modify the response of ph-ORNs to cognate ligands (Deisig et al., 2014) deserves further study if we want to understand the possible ecological and behavioural functions of this physiological process.

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Table 1. Plant blends and individual plant odorants used in the study. Numbers indicate proportion of compounds in each plant blend. Abbreviation is provided for those compounds that were tested individually. * Found in volatile collections of apple and peach, but are not tested behaviourally. References provide behavioural relevance for each compound.

Plant compound	Blend composition			Abbreviation	References
	Chinese	Swiss	Australian		
1-hexanol	1				Lu et al., 2012
nonanal	1			NON	Lu et al., 2012
ethyl butanoate	100			EB	Lu et al., 2012
butyl acetate	70				Lu et al., 2012
ethyl hexanoate	7			EH	Lu et al., 2012
hexyl acetate	5			HA	Lu et al., 2012
hexyl butanoate	1			HB	Lu et al., 2012
farnesene (racemic)	4		100	FAR	Lu et al., 2012; Il'ichev et al., 2009
ocimene (racemic)			100		Il'ichev et al., 2009
(*Z*)-3-hexenyl acetate		100	50	Z3HA	Piñero and Dorn, 2007; Il'ichev et al., 2009
(*Z*)-3-hexenol		20		Z3OH	Piñero and Dorn, 2007
(*E*)-2-hexenal		3		E2A	Piñero and Dorn, 2007
benzaldehyde		20		BZA	Piñero and Dorn, 2007
benzonitrile		0.5		BZN	Piñero and Dorn, 2007
pear ester (ethyl (*E*,*Z*)-2,4-decadienoate)				PE	Knight et al., 2014
citral				CIT	Faraone et al., 2013
3-methylbutyl acetate				3MBA	Lu et al., 2012
terpinyl acetate				TA	Knight et al., 2014
(*E*)-β-caryophyllene				CAR*	Natale et al., 2003
butyl hexanoate				BHX	Lu et al., 2012; Natale et al., 2004
butyl butanoate				BBT*	Lu et al., 2012
octyl acetate				OA*	Wang et al., 2009
methyl salicylate				MS*	Lu et al., 2012
1-octen-3-ol				3OH*	Casado et al., 2006

Table S1. Characteristics of the synthetic plant odorants used in the experiments

Compound	CAS	Company	Prod. num.	Lot number	Purity (%)
1-hexanol	111-27-3	Sigma Aldrich	H13303	TBC5736V	98
1-octen-3-ol	3391-86-4	Aldrich	O5284	PR03904AQ	98
(*E*)-2-hexenal	6728-26-3	SAFC	W256005	19996MH	≥ 95
(*E*)-β-caryophyllene	87-44-5	Fluka	22075	345219/1195	~ 99
(*Z*)-3-hexenol	928-96-1	Fluka	53056	1323459	≥ 98
(*Z*)-3-hexenyl acetate	3681-71-8	Sigma Aldrich	W317101	MKBD9967V	≥ 98
3-methylbutyl acetate	123-92-2	Sigma Aldrich	W205532	MKBC7475V	≥ 97
benzaldehyde	100-52-7	Sigma Aldrich	12010	0001412950	≥ 99
benzonitrile	100-47-0	Fluka	12720	1293869	≥ 99
butyl acetate	123-86-4	Sigma Aldrich	402842	SHBB7070V	≥ 99
butyl butanoate	109-21-7	Aldrich	281964	S02785424	98
butyl hexanoate	626-82-4	SAFC	W220108K	S31491387	>98
citral	5392-40-5	Sigma Aldrich	C83007	STBC5273V	95
ethyl butanoate	104-54-4	Sigma Aldrich	E15701	STBB7416V	99
ethyl hexanoate	123-66-0	Sigma Aldrich	148962	S28172V	≥ 99
farnesene (racemic)	NA	Sigma Aldrich	W383902	MKBF5234V	NA
hexyl acetate	142-92-7	Sigma Aldrich	108154	STBC0601V	99
hexyl butanoate	2639-63-6	Sigma Aldrich	W256803	10311ED-335	> 98
methyl salicylate	119-36-8	Sigma Aldrich	NA	NA	NA
nonanal	124-19-6	Sigma Aldrich	W278203	STBC3506V	≥ 95
ocimene (racemic)	13877-91-3	Sigma Aldrich	W353901	MKBG9855V	≥ 90
octyl acetate	112-14-1	Aldrich	O5500-5G-A	MW05362	> 99
pear ester (ethyl(*E*,*Z*)-2,4-decadionate)	3025-30-7	Sigma Aldrich	W314803	STBC4363V	≥ 80
terpinyl acetate	80-26-2	SAFC	W20470-0-K	06703D407	≥ 95

Table S2. Spontaneous activity (spikes s^{-1}, mean ± SEM) of large, medium and small non-pheromone ORNs housed in sensilla trichodea and auricillica of male *G. molesta*

ORN/sensillum	Sensilla trichodea			Sensilla auricillica		
	Large	Medium	Small	Large	Medium	Small
1	0	0	0	26.20 ± 4.22	0	0
2	03.51 ± 1.80	0	33.27 ± 7.52	09.81 ± 2.75	0	25.13 ± 3.57
3	01.71 ± 1.49	23.85 ± 4.99	19.07 ± 2.80	01.27 ± 0.58	24.51 ± 2.61	33.63 ± 3.56

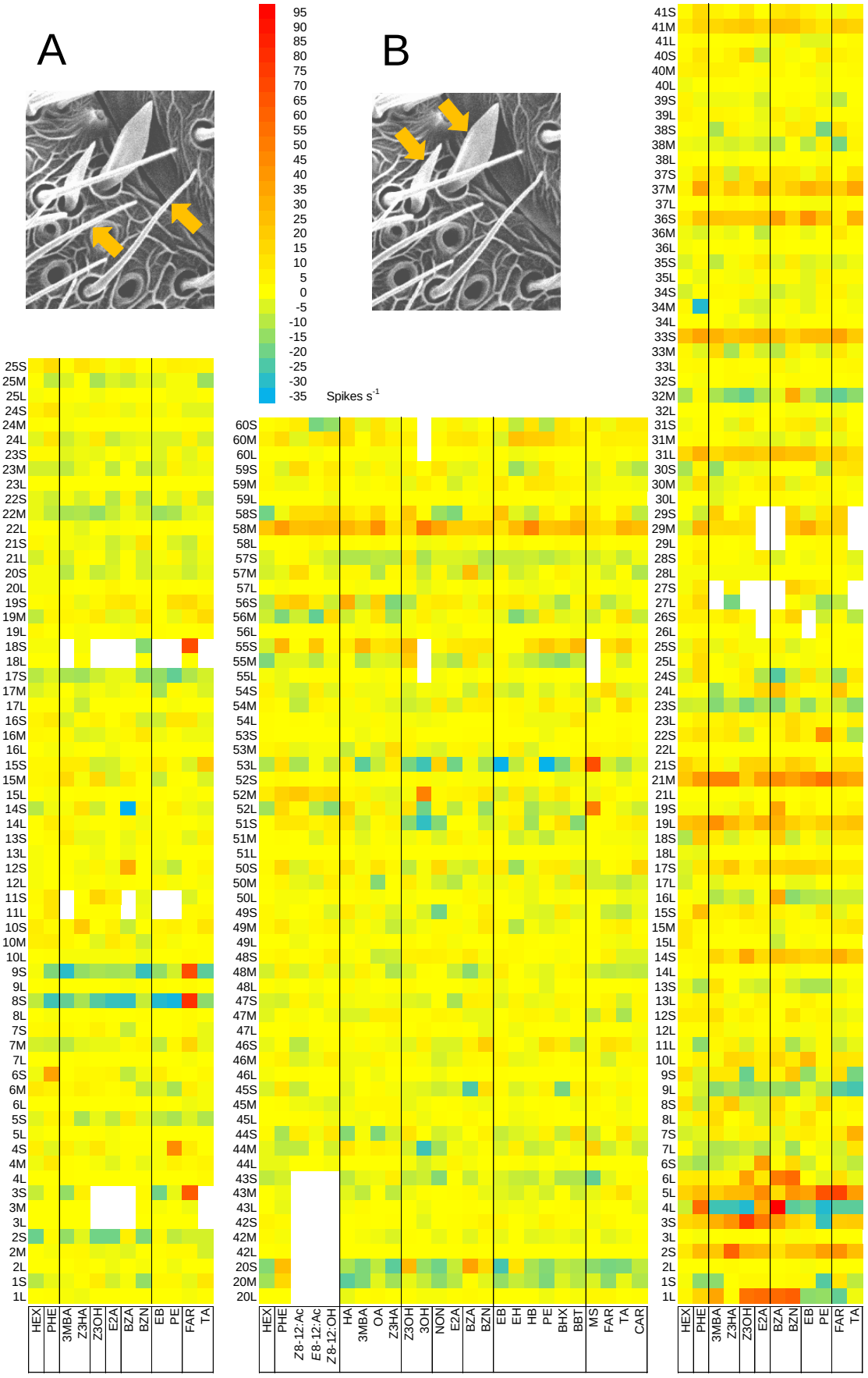
A
B
95
90
85
80
75
70
65
60
55
50
45
40
35
30
25
20
15
10
5
0
-5
-10
-15
-20
-25
-30
-35
Spikes s-1
HEX PHE 3MBA Z3HA Z3OH E2A BZA BZN EB PE FAR TA
HEX PHE Z8-12:Ac E8-12:Ac Z8-12:OH HA 3MBA OA Z3HA Z3OH 3OH NON E2A BZA BZN EB EH HB PE BHX BBT MS FAR TA CAR
HEX PHE 3MBA Z3HA Z3OH E2A BZA BZN EB PE FAR TA

Figure 1. ORN responses to plant volatiles. Sensilla contained between 2 and 3 ORNs characterized by their spike amplitude (L = large, M = medium, S = small). Response intensity is colour-coded according to the accompanying scale bar (spikes s^{-1}, relative to spontaneous activity). Lines group odorants of similar chemical type (*n*-hexane, pheromone, acetates, alcohols, aldehydes, aromatics, esters and terpenoids). A) 64 ORNs from 25 sensilla trichodea were tested with 3 to 10 odorants plus *n*-hexane and the pheromone blend. Maximum responses were -36 and +79 spikes s^{-1}. B) 88 ORNs from 40 sensilla auricillica were tested with 5 to 10 odorants, *n*-hexane and the pheromone blend, and 60 ORNs from 20 sensilla were tested with 19 to 20 odorants, *n*-hexane , the pheromone blend (PHE) and its individual compounds. Maximum responses were -35 and +97 spikes s^{-1}. Most cell responses were very similar to the response elicited by *n*-hexane (yellow range). Little specialization was observed, the most prominent being for racemic farnesene (FAR) occurring in the smaller ORNs of 4 sensilla trichodea (3S, 8S, 9S,18S). In sensilla auricillica, specific odorant responses were observed individual cells (e.g., 52M to 3OH, 32M to BZN, 52L and 53L to MS).

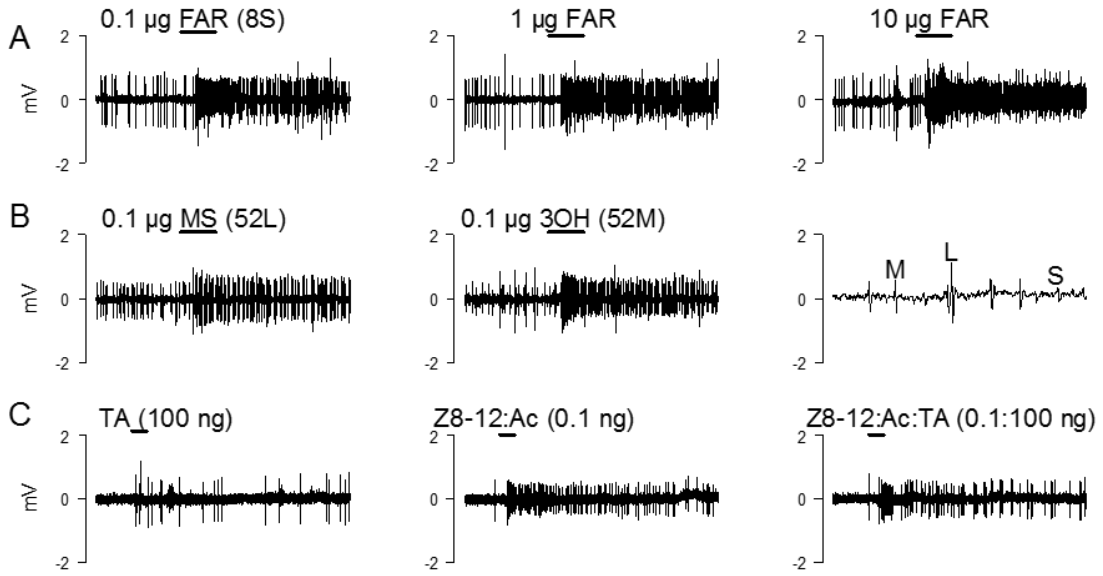

Figure 2. Illustrative SSR traces. A) Sensillum trichodeum housing 2 non-pheromone specialist ORNs, where the response of the smaller amplitude cell to racemic farnesene (FAR) is dose dependent, whereas the larger amplitude cell is unresponsive to this compound. B) Sensillum auricillicum housing 3 ORNs, the large amplitude ORN (left) responds to methyl salicylate (MS) whereas the medium amplitude ORN (middle) responds to 1-octan-3-ol (3OH). A 100 ms trace on the right shows the three ORNs sizes (large [L], medium [M], and small [S]). C) A *Z*8-12:Ac-specific ORN in a sensillum trichodeum is not excited by terpinyl acetate (TA) (left) but its response to *Z*8-12:Ac (middle) is changed by TA (right). Numbers between parentheses correspond to cells shown in Fig. 1. The horizontal bar above each trace represents stimulus duration (200 ms).

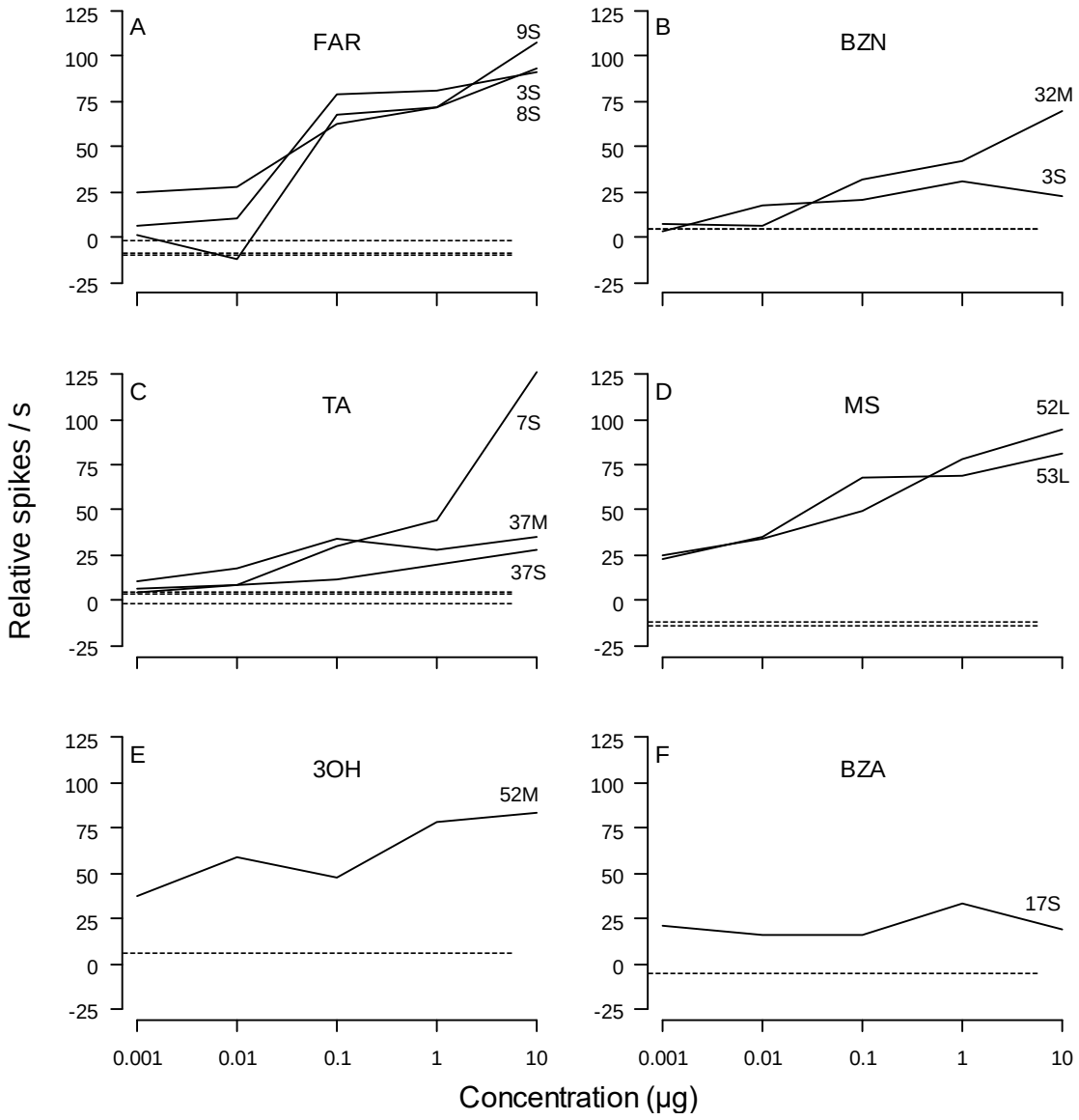

Figure. 3. Dose-response curves of non-pheromone-specific ORNs housed in sensilla trichodea (A) and auricillica (B-F) of *G. molesta* males. Dotted lines are the ORNs response to *n*-hexane. Numbers correspond with ORNs shown in Fig. 1.

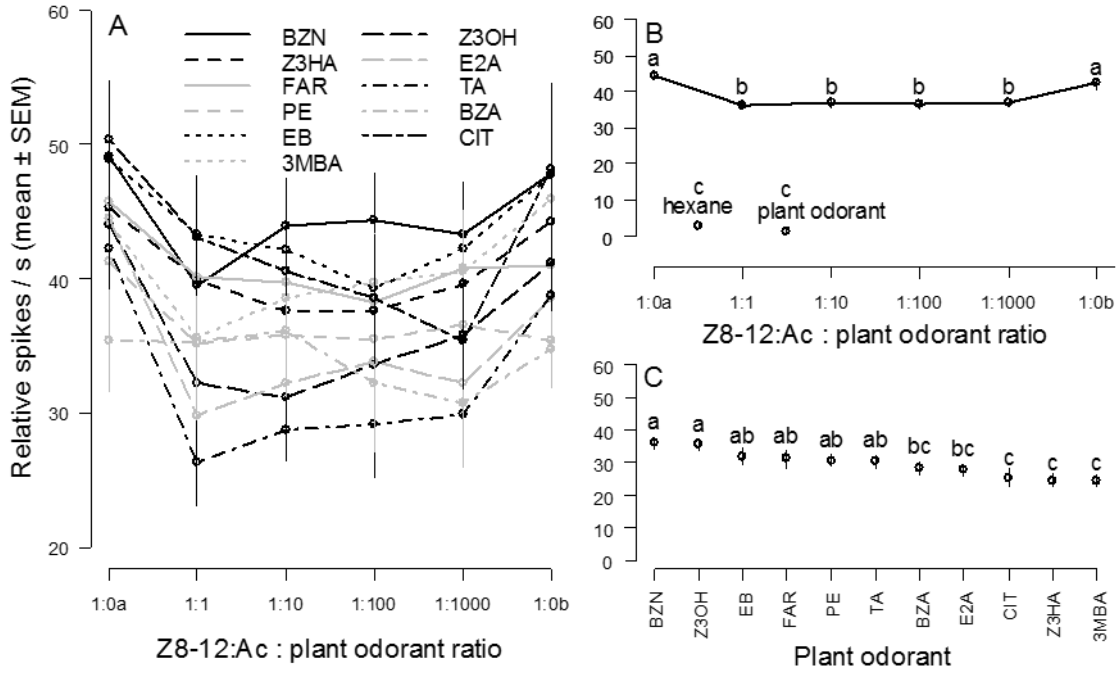

Figure 4. Effect of plant odorants on the response of *Z*8-12:Ac ORNs to sex pheromone. ORNs (n = 8) were stimulated with *Z*8-12:Ac alone at 0.1 ng, *Z*8-12:Ac mixed with the plant odorant in 1:1 to 1:1000 pheromone: plant-odorant ratios (*Z*8-12:Ac at 0.1 ng), and a second 0.1 ng puff of *Z*8-12:Ac. A) Average response for each plant odorant and dose combination. B) *Z*8-12:Ac ORNs were not responsive to *n*-hexane or the plant odorants, but their response to *Z*8-12:Ac decreased when it was mixed with the plant odorant at all the doses. C) Some odorants caused more inhibition than others. Means in C are lower than in A and B because *n*-hexane and plant odorants are pooled in C. Different letters in B and C indicate significant differences among treatment means (Tukey pair-wise comparison after GLM, $P < 0.05$).

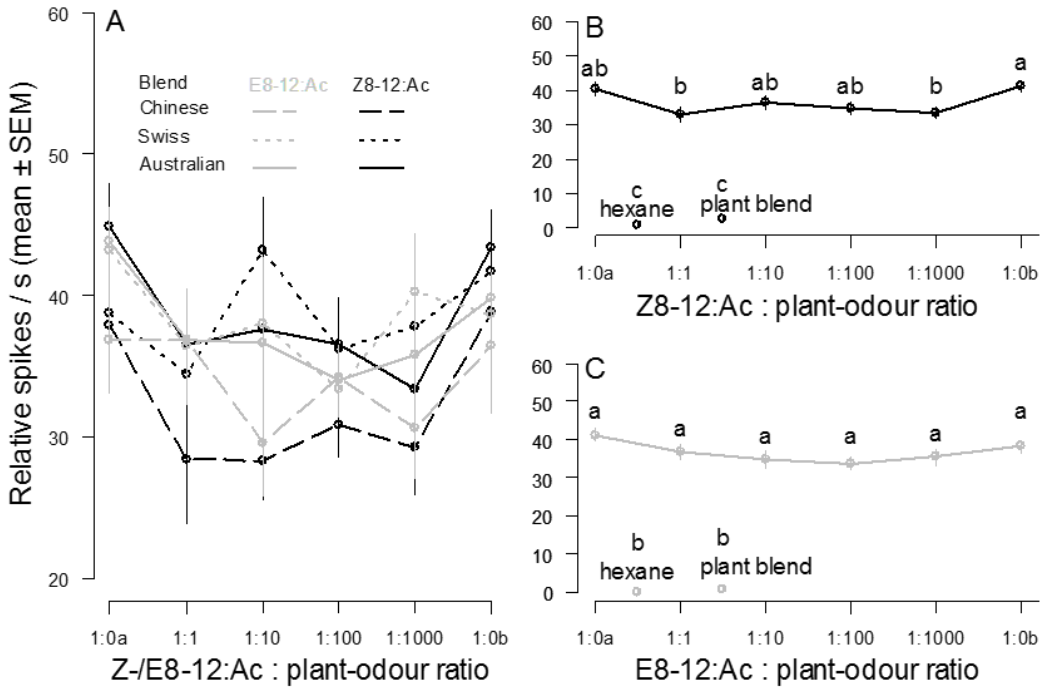

Figure 5. Effect of plant odors (i.e., blends) on the response of pheromone-specific ORNs to sex pheromone and pheromone-plant blends. *Z*-ORNs (n = 8) and *E*-ORNs (n = 5) were stimulated with *n*-hexane, one of 3 plant blends (Australian, Chinese and Swiss, 100 ng), the pheromone ligand alone (*Z*8-12:Ac or *E*8-12:Ac ,0.1 ng, 1:0a), the pheromone ligand mixed with the plant odorant in 1:1 to 1:1000 pheromone: plant-odor ratios (pheromone at 0.1 ng), and a second 0.1 ng puff of the pheromone ligand (1:0b). A) Average response for each plant odor and dose combination. B) The response of *Z*-ORNs to *Z*8-12:Ac was reduced by the plant odors at pheromone:plant doses of 1:1 and 1:000. C) The response of *E*-ORNs to *E*8-12:Ac was not affected by the plant blends. Different letters indicate significant differences among treatment means (Tukey pairwise comparison after GLM, $P < 0.05$).

GENERAL DISCUSSION

General Discussion

Here I discuss the effect of behaviorally active plant blends combined with suboptimal pheromone blends on the response of male *G. molesta* both in laboratory and field tests. Further I also discuss the mechanisms of pheromone plant interaction emphasizing the antennal ORNs level.

1. Effect of plant blends on behavioral response

In these studies I compared Australian, Chinese and Swiss plant blends side-by-side simultaneously in the same location in Chile, and I found that none of the plant blends tested are attractive to *G. molesta* males on their own, neither in field trap tests, nor in the flight tunnel tests. In contrast, these plant blends are behaviorally active (Pinero and Dorn, 2007), and attract both male and female *G. molesta* on their own in the field (Il'ichev et al., 2009; Lu et al., 2012). Poor responses of moths to synthetic plant volatile lures is not surprising given the difficulty to reproduce artificial lures that can compete with natural host blends under a background of volatile odors in the wild. For example, although, pear ester is a successful attractant for *C. pomonella*, it performs very differently in different crops and locations (Knight, 2010; El-Sayed et al., 2013). Addition of plant odors of different origin synergised male responses to the sub-optimal doses of pheromone in the flight tunnel. My findings of Swiss plant blend synergism are in accordance with a previous study on *G. molesta* (Varela et al., 2011a), and support other moth studies of pheromone-plant synergism (Reddy and Guerrero, 2004). In contrast, the same plant stimuli that were synergistic in the wind tunnel, were inhibitory in the field. Similar findings are reported in in at least one other moth (Meagher and Mitchel, 1998; Meagher, 2001).

There is increasing realization that background odors in the environment influence the response of insects to pheromone and plant stimuli (Bruce et al., 2005; Reinecke and Hilker, 2014), so background odors could explain the disagreements among between my study and studies in Australia (Il'ichev et al., 2009) and China (Lu et al., 2012). High pheromone:plant ratios (1:100 and 1:1000) synergize the response of *Plutella xyllostella* (L.) (Dai et al., 2008), which supports that the ratios that I tested are in the normal range of moth detection. However, a recent study shows that low pheromone:plant ratios (1:1 and 1:2) increased male *G. molesta* captures in the field (Yu et al., 2014). At a 1:10 pheromone:plant ratio, green leaf volatiles synergize the attraction of *C. pomonella*, *Heliothis zea* (Boddie) and *Heliothis virescens* (F.) to sex pheromone (Dickens et al., 1993; Light et al., 1993). Clearly, the pheromone:plant ratio seems a critical aspect to take into consideration in pheromone-plant studies because insects could be attracted or repelled by the same plant volatile depending on the dose. The difference between the Australian and Chinese studies and the Chilean study could result from the characteristics of the stimulus itself (dispenser, concentration, chemical purity, trap type), the genetic architecture of the population, and the composition of background odors. All these factors should be taken into consideration because all of them have been shown to play a role in shaping insect response to plant volatiles.

Male *G. molesta* responses decreased with increase in the pheromone concentration, as reported previously (Varela et al., 2011a), however in the present study I failed to observe plant synergism to an over-dose pheromone blend. This could be explained by the different mechanisms by which low and high pheromone doses reduced response levels. With low doses the olfactory system is under-stimulated and therefore the stimulus arriving to the CNS is probably below the behavioral response threshold. Plant odors, which in my test did not stimulate male flight on their own but that under natural conditions could signal the presence of conspecific females (Deisig et al., 2014), may lower the behavioral response threshold to pheromone (since the pheromone receptor neurons are unaffected by the presence of plat odors in the pheromone blend, Ammagarahalli and Gemeno, 2015), and so increase responses to below optimal pheromone doses. With high stimulus doses however, the olfactory system is sufficiently stimulated from the distance to arouse take flight and oriented flight, but males interrupt upwind progress (i.e., arrest) close to the odor source probably due to adaptation at the peripheral olfactory level (De Bruyne and Baker, 2008). Under these conditions the effect of the plant odor is probably negligible, given that the pheromone receptor neurons are probably adapted and unable to transmit a proper pheromone stimulus to the brain despite simultaneously processing an optimal plant signal. Schmidt-Büsser et al. (2009) report behavioral synergism to an overdose pheromone blend in the tortricid *Eupoecilia ambiguella* Hübner, so in some cases the plant blend can cancel out the effect of a high pheromone dose, but more studies are needed to have a broader picture of this phenomenon.

The wind tunnel study shows that changes in the optimal proportion of *E*8-12:Ac in the pheromone blend can alter male *G. molesta* responses drastically. My findings are in agreement with similar studies (Linn and Roelofs, 1981; Willis and Baker, 1988; Knight et al., 2015) where the *E*8-12:Ac proportion is critical for optimal male attraction. Here I tested unnatural ratios of *E*8-12:Ac in the pheromone blend combined with increased concentrations of plant blend. Addition of plant blend reverted some of the negative effects of the unnatural low and high E-isomer ratios, mainly at the earlier stages of response (take off and oriented flight), but it failed to influence the response of the male when close to the pheromone source. These results suggest that although plant odors are able to compensate for unnatural pheromone blend ratios under laboratory conditions, the effect may be diluted under field conditions where plant volatiles are ubiquitous and therefore will mix with the pheromone stimulus (Deisig et al., 2014).

The alcohols 12:OH and codlemone produced the same effect as *Z*8-12:OH when this compound was removed from the blend, so this alcohol does not appear to be an essential ingredient in the pheromone blend of *G. molesta* since its role can be replaced by similar compounds. Cardé et al. (1975a,b) report that 12:OH acts only at the close-range, however I found that it significantly improved all the stages of the behavioral male response in the wind tunnel. A field test would be needed to confirm long-range responses to this compound under natural conditions. Baker and Cardé (1979) indicate that the role of the two alcohols (*Z*8-12:OH and 12:OH) depends on the presence of each other and on

the ratio of *E*8-12:Ac to *Z*8-12:Ac in the blend, so further tests with more treatment combinations may show additional roles for these alcohols. *G. molesta* is not attracted to the sex pheromone of *C. pomonella*, *E*8,*E*,10-12:OH, but when this compound is mixed with its own pheromone it increases *G. molesta* captures, and their combined use is a new approach targeting both populations in the field (Evenden and McLaughin, 2005; Knight et al., 2014). In my test, unlike the previous ones, the effect of codlemone was tested in the absence of *Z*8-12:OH, and I show that codlemone effected similar levels of synergism as *Z*8-12OH, and interestingly both had the strongest effect at the 10% ratio.

The three alcohols have relatively similar chemical structures, so a generalistic alcohol ORN could be enough to detect the three of them. On the other hand, each alcohol molecule could have its own specific receptor. I have been unable to detect ORNs on the male antenna that are specifically tuned *Z*8-12:OH in the sampled sensilla (Chapter 3 of this thesis, Ammagarahalli and Gemeno, 2014), but whether there are receptors tuned to codlemone or 12:OH remains to be tested. It is unlikely, though, that male *G. molesta* would have a receptor specific for codlemone because the two species do not cross-attract as they do not share the main pheromone compounds (Knight et al., 2014). Plant volatiles do not excite pheromone ORN, instead they are perceived by general odor ORNs housed in other sensilla, mainly auricillica but also in some sensilla trichodea (Ammagarahalli and Gemeno, 2015). The synergism of the plant blend on the no-alcohol pheromone blend may involve excitation of these plant ORNs.

Support for the importance of *Z*8-12:OH as an ingredient in the pheromone of *G. molesta* comes from studies showing that calling females release it (Baker et al., 1980), that males do not respond to a Z-and E8-12:Ac blend and that just a small percentage of Z8-12:OH (1-3%) is needed to increase male attraction significantly (Baker and Cardé, 1979; Linn and Roelofs, 1983). By contrast, other studies indicate that *Z*8-12:OH is not necessary for attraction (Roelofs and Cardé, 1974; Yang et al., 2002), its proportion in the blend can vary widely without affecting male response (Linn and Roelofs, 1983), and females do not release it (Lacey and Sanders, 1992). Pheromone composition and male response are very consistent across worldwide populations of *G. molesta* (Knight et al., 2014), but in comparison, other studies show little or no traces of *Z*8-12:OH in the female gland extractions (reviewed by Boo, 1998; El-Sayed and Trimble, 2002).

Closely-related species sharing similar pheromone blends, and therefore at risk of interspecific mating, may evolve olfactory signals designed to deter mutual attraction (Cardé and Haynes, 2004). *Z*8-12:OH inhibits males of two species that are closely related and that use a similar ratio of the Z/E acetates as main pheromone ingredients as *G. molesta* [*Grapholita funebrana* (Treitschke) (Guerin et al., 1986), and *Grapholita prunivora* (Walsh) (Baker and Cardé, 1979)], so it is possible that the production and release of *Z*8-12:OH by *G. molesta* females may serve an interspecific avoidance function. In a similar fashion, two compounds in the pheromone glands of *G. funebrana* (Z8-14:Ac and Z10-14:Ac) do not play a role in attracting this species but they reduce captures of *G. molesta* (Guerin et al., 1986). Interestingly lesser captures of *C. pomonella* to mixture of the two pheromone blends is reported (Knight et al., 2014).

2. Effect of plant blends on physiological responses

In the behavior studies I show that plant blends synergize male *G. molesta* responses to under-dosed pheromone. Then I asked if this behaviorally synergism occurs in the ph-ORNs. I characterized ph-ORNs of male *G. molesta* which have not been characterized in the past. Scanning electron microscopy of antennae revealed six different types of sensilla on the flagellomeres: trichodea, chaetica, coeloconia, auricillica, basiconica and styloconica. The different types varied in distribution and density along the flagellum and between scaled and scale-free areas, and are similar to those reported in other tortricids (Wall, 1978; Razowski and Wojtusiak, 2004; Ansebo et al., 2005), and Lepidoptera in general (Hansson, 1998). Single sensillum recording technique was used to characterize the response of ORNs housed in the most abundant sensilla trichodea, which usually respond to pheromone components. Based on the response profile to pheromone stimuli, sensilla trichodea were grouped in to three distinct groups: those housing ORNs tuned specifically to the major component (*Z*8-12:Ac), others housing ORNs tuned to minor component (*E*8-12:Ac), and sensilla with ORNs that did not respond to any of the three pheromone components. The proportion of ORNs tuned to the major and minor pheromone components were similar to their share in the pheromone blend. However, I did not find a ORN type specific to *Z*8-12:OH in the sensilla trichodea sampled. The apparent absence of *Z*8-12:OH-specific ORNs, although this compound is emitted by females and affects male attraction (Cardé et al., 1975; Cardé et al., 1979; Baker and Cardé, 1979; Baker et al., 1980; Linn and Roelofs, 1983), could be explained if only few ORNs are dedicated to *Z*8-12:OH and they were missed in our sampling. Stimulation with *Z*8-12:OH to ORNs housed in sensilla auricillica reveal no alcohol-specific types. In contrast, sensilla auricillica ORNs respond to pheromone components in *C. pomonella* (Ansebo et al., 2005).

In *G. molesta*, ORNs tuned to *Z*-and *E*8-12:Ac are housed in different sensilla trichodea unlike in other tortricids and many other moths, where pheromone ORNs are co-localized in the same sensillum (reviewed in De Bruyne and Baker, 2008; Baker et al., 2012). It has been proposed that the adaptive function of co-localized ORNs is related to the physiological constraint imposed by real time detection of precise odorant blend ratios (Baker et al., 1998; Baker et al., 2012; Binyameen et al., 2014). As a general rule, when ORNs are co-localized the major component ORN has a larger dendrite size, whereas sensilla with a single ORN responding to the major component are more abundant than sensilla housing neurons tuned to minor components (Baker et al., 2012). The second case applies to *G. molesta* because major and minor component ORNs occur in different sensilla, and the major component ORNs are more abundant than minor component ORNs, whereas the spike amplitude of both ORN types is relatively similar, which is an indication of similar dendrite size between them (Hansson et al., 1994).

The large percentage of pheromone unresponsive ORNs housed in sensilla trichodea was surprising. Several studies in other moth models also report the presence of ORNs unresponsive to the pheromone components (Hansson et al., 1989; Kalinová et al., 2001; Baker et al., 2004). After stimulating pheromone unresponsive ORNs with plant odors I found that most of them did not respond to plant odors either. However one ORN

type responded to racemic farnesene (6 % of unresponsive ORNs). A large percentage (probably more than 50 %) of ORNs housed in sensilla auricillica could be considered unresponsive to the plant stimuli panel that I have tested, and indeed very little specialization was recorded. Other tortricid moth study report similar percentages of unresponsive ORNs in sensilla auricillica (Ansebo et al., 2005), so it appears that non-responding ORNs are not uncommon, but the reasons for such widespread ORNs "silence" are still unclear. One possible explanation for the high number of unresponsive ORNs is that many plant ORNs have narrow molecular receptive ranges (i.e., they are specialist), and that if the odor panel with which they are stimulated is modest (like the one I have tested), then only a few of them will show specific responses. A Concert of excitatory and inhibitory responses to different compounds by the same ORN may increase the coding capability of plant-ORNs and help insects decode a diverse plant stimulus landscape using less ORNs than if only excitatory responses were produced (Bruce and Picket, 2011; Clifford and Riffell, 2013). Specialist plant ORNs are, indeed, common in moths (De Bruyne and Baker, 2008), and in some cases they appear to be relatively abundant, such as the 30 % responding to phenyl acetaldehyde in *S. littoralis* (Binyameen et al., 2012).

I explored if the pheromone and plant odor synergism observed in behavior is happening at the peripheral ph-ORNs. The response of ph-ORNs was lower to a mixture of pheromone and plant stimuli than to a pheromone ligand alone. The slight inhibitory effect was consistent in the case of *Z*8-12:Ac ORNs tested with plant odorants, but sporadic or absent in the case of *Z*- and *E*8-12:Ac ORNs tested with plant odour blends. Ph-ORNs did not respond to any of the plant volatiles tested alone. Inhibitions of responses of ph-ORNs are also reported in other moths (Deisig et al., 2012; Hillier and Vickers, 2011). It is intriguing, though, that with few exceptions (Ochieng et al., 2002; Hillier and Vickers, 2011; Rouyar et al., 2015), in the majority of species where it has been tested, including *G. molesta*, the effect of plant volatiles is to decrease (and not increase) the response of ph-ORNs to pheromone (Deisig et al., 2014). This is counterintuitive because if pheromone-plant stimuli integration at the ph-ORN level were to explain behavioral synergism, one would expect that the mix would increase, and not decrease, ph-ORN responses to pheromone. A possible physiological function of pheromone suppression by plant volatiles is to improve pheromone pulse resolution, and thus potentially aid male orientation to pheromone-emitting females (Party et al., 2009; Deisig et al., 2014), although this remains to be tested with free-flying insects. In contrast, most available evidence indicates that pheromone and plant stimuli travel *via* separate nerve lines to the AL and that integration takes place in there (Christensen and Hildebrand, 2002; Lei and Vickers, 2008; Namiki et al., 2008), so sensory integration at the peripheral level seems even more redundant. However, in the tortricids *C. pomonella* and *G. molesta* some projection neurons responding to pheromone innervate ordinary glomeruli and not the MGC located at the entrance of the antennal nerve, which typically receives pheromone input from the antenna (Trona et al., 2010, Varela et al., 2011b). This unusual pattern of coding in the AL could be explained by the response of non-pheromone

ORNs to both pheromone and plant compounds at the peripheral receptor level in *C. pomonella* (Ansebo et al., 2005), or even in *G. molesta*, as I have shown. To conclude, the observed behavioral pheromone-plant synergism in male *G. molesta* probably does not start in antennal ORNs.

A great amount of knowledge on the olfactory processing of *G. molesta* at the peripheral level has been achieved in the work, but the results also raise new questions. Though *Z*8-12:OH was known to be important in the behavior, no ORN was tuned to it, which suggests the need of vast exploration of ORNs tuned to *Z*8-12:OH and other alcohols on the antennae. There is a need of exploration of AL neurons to find the mechanism of pheromone and plant odour synergism in the behaviour.

CONCLUSIONS

Conclusions

Under natural conditions sex pheromones and plant odors mix in the air and together stimulate responding insects, however relatively little is known about the effect of plant odors on pheromone response. Plant blends synergize male moth behavioral response, including *G. molesta*. However, synergism of the pheromone-plant blend has not been tested in either field or laboratory conditions except Swiss blend in the laboratory. Mixture of under-dosed pheromone and increasing doses of tested plant blends (i.e. Australian, Chinese and Swiss), significantly synergize male *G. molesta* responses compared with the under-dosed pheromone blend alone in the wind tunnel. On the other hand, plant blends alone attract no males in either wind tunnel or field conditions. By contrast, plant blends combined with pheromone decreased male captures in the field. Inhibitory responses in the field could be due to high ratio of plant blend to pheromone in the mixtures tested. Although the Australian and Chinese plant blends were relatively successful in attracting *G. molesta* in the original studies, when tested side-by-side in Chile did not attract any males, although I reproduced the experimental conditions of the original studies. Possible explanations for poor responses could be the result of composition of background odors, characteristics of stimulus, or genetic variation in the populations. In addition, our tests were not exhaustive.

Several studies in both field and laboratory conditions show that plant blends synergize male moth responses to sex pheromone, including *G. molesta*. However, few studies have explored the effect of plant odors on male response to unnatural pheromone composition or unusually high concentrations. Our laboratory study shows that plant odors cannot minimize the reduction of male *G. molesta* responses to high-doses of pheromone blend. In addition, plant odors can also counteract decreased male responses to unnatural pheromone component ratios. Additionally, plant odors and other alcohol pheromone components can replace the absence of *Z*8-12:OH, in the pheromone blend. Thus, plant odors and other alcohol components provide some flexibility to the pheromone response of male *G. molesta*. These findings pose new questions regarding perception and integration of pheromone and plant signals, and future studies should explore how the olfactory system perceives and integrates plant and pheromone information in order to understand the interplay between these two types of stimuli.

Although behavioral studies show that plant odors synergize male *G. molesta* responses to under-dosed pheromone blend, the physiological mechanisms of pheromone-plant synergism are not well understood. It may occur already at antennal ORN level. To test this in *G. molesta* first I mapped and calculated the abundance of different types of sensilla located on the antennae using scanning electron microscopy. I used single sensillum recording technique to characterize the response of ORNs stimulated with pheromone and plant odors. A large proportion of the sensilla on male moth antennae are sensilla trichodea housing ORNs tuned to the pheromone components. The ratio of Z and E ph-ORNs in the antenna is similar to the ratio of each pheromone component in the blend released by conspecific females. *Z*8-12:Ac ORNs are highly specific, whereas *E*8-12:Ac ORNs also respond to *Z*8-12:Ac, but with lower sensitivity. In male moths the

ORNs of each pheromone component are either located in the same sensillum or in separate sensilla. *G. molesta* males belong to the second type because *Z*8-12:Ac and *E*8-12:Ac ORNs occur in different sensilla trichodea. Apparent absence of Z8-12:OH-specific ORN could be due to an insufficient sample size of a very uncommon neuron type. I found that 30 % of the sensilla trichodea do not house ph-ORNs and some of them were tuned to the plant odorants. Finding no ph-ORNs housed in the sensilla trichodea is not uncommon in other tortricids.

To determine if the behavioral pheromone-plant synergism occurs in the ph-ORNs, I estimated behaviorally realistic doses of pheromone and plant odorants based on the dose-response curves. Ph-ORNs did not respond to the plant odors and odorants tested. Stimulation of Z-ORNs with binary mixtures of plant odorants and *Z*8-12:Ac reduced their responses subtly, but the effect was concentration-independent. The response of *E*-ORNs to a combination of *E*8-12:Ac and plant volatiles was not different from *E*8-12:Ac alone. I argue that the small inhibition of *Z*-ORNs caused by physiologically realistic plant volatile doses is probably not fully responsible for the observed behavioral synergism of pheromone and plant odors. I conclude that the observed behavioral pheromone-plant synergism could start in the antennal lobe neurons, like in other moths.

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